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The Preparation and Characterization of Component C of
Methylococcus capsulatus (Bath)

by

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Thesis submitted for the degree of Doctor of Philosophy

University of Warwick

Department of Biological Sciences

November 1983

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SUMMARY

The methane monooxygenase (MMO) present in soluble extracts of Methylococcus capsulatus (Bath) can be resolved into three components, called protein A, protein B, and protein C. Protein C, the subject of this thesis, is a polypeptide chain with one FAD and one iron-sulphur centre per molecule, and is the reductase component of the monooxygenase system, for which NADH is the reducing substrate. Protein C transfers reducing equivalents to protein A, the monooxygenase component, responsible for substrate hydroxylation.

The aim of this project was to investigate how electrons were transferred from NADH to protein A, and work focused on the 2 redox centres of protein C.

(1) Protein C has been prepared and purified by a modified method, which is quicker and more reproducible than published methods.

(2) A critical comparison of present and previous characterisation work on protein C has been undertaken.

(3) The three mid-point potentials of the protein C redox centres have been determined and shown to be consistent with their ability to transfer electrons to protein A from NADH; additionally, the optical contributions of the redox centres in various states of reduction have been resolved.

(4) The order of electron flow in the two redox centres has been shown to be, NADH ---> FAD ---> FeS ---> protein A, by selective removal and reconstitution of the centres.

(5) With saturating NADH, it has been shown that NADH interaction and reduction of protein C, and electron transfer to protein A, are not rate-limiting to the overall MMO reaction, and electron transfer to protein A is independent of protein B, which is proposed to act after this step; a gross kinetic mechanism is proposed.

(6) Possible technological uses of protein C are explored, based on the ability of protein C to be reduced by redox dyes, to regenerate NADH, and to reduce the redox electrode without mediators.

For Theo. Anne, Dot, Ginny, Rosy, and Marc.

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Declaration

The material presented in this thesis is, to the best of my knowledge, the result of original research conducted by myself, under the supervision of Prof. H. Dalton, apart from:-

work involving pure proteins A and B (4.3) performed in collaboration with Dr. M.P. Woodland; the recording of the EPR spectra (3.1.C.), performed by Dr. D.J. Lowe; some of the work on the effects of ethene on the activity of soluble extracts, carried out in conjunction with Dr. S. Stanley (2.1.B.); and some stability studies carried out in collaboration with Mr S.J. Pilkington (2.3.C.) (all indicated in the text). None of this work has been submitted for examination previously.

ABBREVIATIONS

| | |
|-----------|---|
| A | Absorbance |
| Å | Angstrom |
| AMP | adenosine monophosphate |
| BSA | bovine serum albumin |
| dansyl | 5-dimethylamino-1-naphthalene sulphonyl chloride |
| DCPIP | 2,6,-dichlorophenolindophenol |
| DEAE | diethylaminoethyl |
| DNase | deoxyribonuclease |
| E | extinction coefficient |
| E_m | mid-point potential |
| e | electron |
| EDTA | ethylenediaminetetra-acetic acid |
| EPR | electron paramagnetic resonance |
| ESR | electron spin resonance |
| FAD | flavin adenine dinucleotide |
| FMN | flavin mononucleotide |
| IEF | isoelectric focusing |
| IgG | Immunoglobulin G |
| K | Kelvin |
| K_D | dissociation constant |
| K_{eq} | equilibrium constant |
| K_i | inhibition constant |
| K_m | Michaelis-Menten constant |
| k | rate constant |
| k_{cat} | turnover rate constant |
| k_{red} | rate constant for reduction |

| | |
|-----------------|--|
| MMO | methane monooxygenase |
| mA | milliamperes |
| mV | millivolts |
| n | number of electrons transferred |
| NAD | nicotinamide adenine dinucleotide |
| NADD | (4S)-[² H] NADH |
| NADH | reduced nicotinamide adenine dinucleotide |
| NADP | nicotinamide adenine dinucleotide phosphate |
| NADPH | reduced nicotinamide adenine dinucleotide phosphate |
| NBT | nitro blue tetrazolium |
| O.D. | optical density |
| PAGE | polyacrylamide gel electrophoresis |
| pI | isoelectric point |
| pO ₂ | partial pressure of oxygen |
| S | substrate concentration |
| S | Svedburg coefficient |
| SDS | sodium dodecyl sulphate |
| T | temperature in degrees centigrade |
| TEMED | N,N,N',N'-tetramethylenediamine |
| TRIS | tris(hydroxymethyl)aminomethane |
| unit | micromole per minute per milligram |
| v | reaction rate |
| . | unpaired electron |

CHAPTER 1. Introduction

1.1. General Introduction

1.1.A. The occurrence and isolation of methane-utilizing bacteria

Methane is produced naturally in a number of diverse situations; coal and oil deposits, the rumens of ruminant animals and in lakes and ponds (Table 1, Ehhalt, 1976). Methane-utilizing bacteria are usually isolated from lakes or ponds. The anaerobic decomposition in the sedimented silt layer at the bottom of lakes and ponds gives rise to methane; but due to the oxygen requirement of methane-utilizing bacteria, these organisms are rarely found in the anaerobic region of the sediment. All methane-oxidizers are strictly aerobic due to a requirement for gaseous oxygen for the initial oxidation of methane. Methane-oxidizers grow primarily just above the anaerobic environment in areas of low oxygen tension, forming a concentration layer of methane-oxidizing activity (Cappenberg, 1972). In experiments with columns of sediment, in some cases none of the methane generated by methanogenic bacteria in the lower anaerobic regions is released into the atmosphere above the sediment, indicating that methane-oxidizers can be highly effective in utilizing methane (Whittenbury et al., 1976). The presence of methane-oxidizers has been known since the turn of the century, when Söhnngen isolated Methylomonas methanica, a bacterium capable of growth on methane and methanol as the sole carbon and energy source (Söhnngen, 1906). This was one of the three species of methane-utilizing bacteria which had

Table 1. Sources of atmospheric methane.

| Source | Global methane production (Mt/year) |
|---------------------------------|--|
| Biogenic* | |
| Enteric fermentation in animals | 101-220 |
| Paddy fields | 280 |
| Swamps, marshes | 130-260 |
| Freshwater lakes | 1.3-2.5 |
| Upland fields | 10 |
| Forests | .4 |
| Tundra | .8-8 |
| Oceans | |
| open | 4-6.7 |
| continental shelf | .07-1.4 |
| Total biogenic | <u>528-812</u> |
| Other sources | |
| Coal mining | 6.3-22 |
| Lignite mining | 1.6-5.7 |
| Industrial losses | 7-21 |
| Automobile exhaust | .5 |
| Volcanic emissions | .2 |
| Total all sources | <u>544-862</u> |

* These figures indicate only methane released into the atmosphere and do not include biogenic methane oxidised by methane-utilizers.

been isolated and characterized up until 1970, when Whittenbury and his colleagues isolated more than a hundred methane-utilizing bacteria. These workers overcame the isolation problems due to the relatively slow growth of methane-utilizers as compared with the growth of the large numbers of non-methane-utilizing contaminants. Samples were enriched in liquid culture and serially diluted onto solid media, instead of proceeding by a direct plating method, and colonies were isolated whilst still at the microscopic stage (Whittenbury et al., 1970). One of these isolates was the Bath strain of Methylococcus capsulatus, which is the subject of study in this thesis. This strain was obtained from the warm water springs of Bath and was described as a strictly aerobic Gram-negative non-motile coccus (Figure 1).

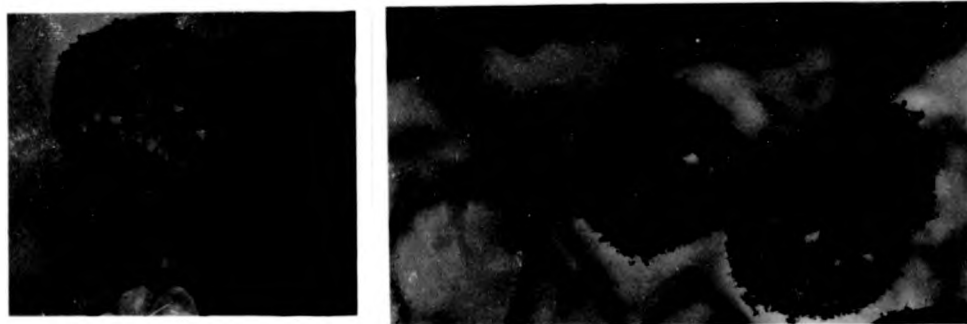
1.1.B. The methane oxidation pathway

The pathway for methane oxidation has been generally assumed to involve the following series of reactions:-



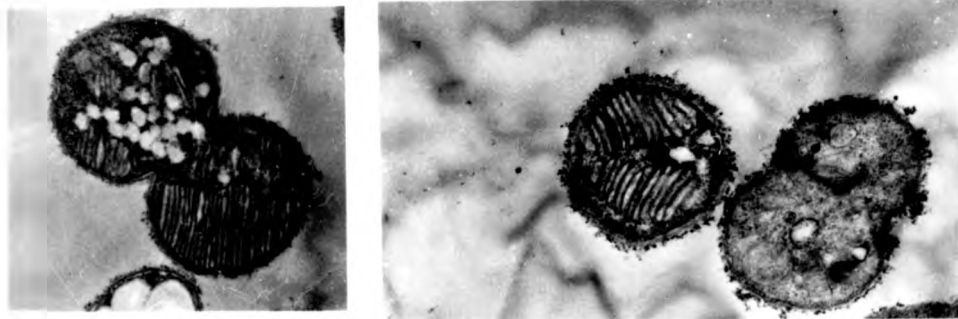
The evidence in favour of this pathway, originally proposed by Dworkin and Foster in 1956, is not complete for any one organism, but the proposed scheme is the simplest one to account for the known facts (see Quayle, 1972, for a review). In all methane-oxidizers tested it appears that cellular carbon is assimilated at the level of formaldehyde either by a serine or a ribulose monophosphate pathway, the

Figure 1. Thin section electron micrographs of *Methylococcus capsulatus* (Bath) (x 20,000).



The presence of stacks of intracytoplasmic membranes appears to correlate with the occurrence of the particulate methane monooxygenase, whereas the soluble methane monooxygenase correlates with a lack of these membrane arrays (S.D.Prior, personal communication). These prints were provided by S.J.Pilkington.

Figure 1. Thin section electron micrographs of *Methylococcus capsulatus* (Bath) ($\times 20,000$).



The presence of stacks of intracytoplasmic membranes appears to correlate with the occurrence of the particulate methane monooxygenase, whereas the soluble methane monooxygenase correlates with a lack of these membrane arrays (S.D.Prior, personal communication). These prints were provided by S.J.Pilkington.

energy for assimilation being derived from the further oxidation of formaldehyde to carbon dioxide (see Anthony, 1975 for review).

The proposed intermediates methanol, formaldehyde and formate have been shown to be oxidized by whole-cell suspensions of various methane-utilizing bacteria, and methanol can act as an alternative carbon source to methane.

This has also been shown for Methylococcus capsulatus (Bath) (D.J.Leak and S.H.Stanley, personal communication). The three proposed intermediates have been detected separately during the oxidation of methanol and methane in various bacteria, under conditions of environmental stress. For Methylococcus capsulatus (Bath) methanol has been detected during methane oxidation in the presence of ethanol (S.H.Stanley, personal communication). In various bacteria the enzymes which catalyze the oxidation of methane, methanol, formaldehyde and formate have been found in cell-free extracts and purified (see Anthony, 1982 for review). This has been demonstrated with Methylococcus capsulatus (Bath) for the soluble methane monooxygenase (Colby and Dalton, 1976, 1978, 1979; Dalton, 1980), for the formaldehyde dehydrogenase (Stirling and Dalton, 1978), and the methanol dehydrogenase has been partially purified in this laboratory (S.H.Stanley, personal communication). Therefore satisfactory evidence exists that the pathway proposed by Dworkin and Foster holds for Methylococcus capsulatus (Bath).

1.2. The methane oxidation reaction

1.2.A. Oxygen involvement in the methane oxidation reaction

Early studies on the oxidation of methane were concerned with the origin of the oxygen atom present in methanol. The first indirect evidence for the involvement of gaseous oxygen in the initial methane oxidation step was obtained in 1959 by growing Methylobionas methanica in the presence of $^{18}\text{O}_2$. Sixteen fold more label was incorporated into cell material using methane as the carbon source rather than methanol (Leadbetter and Foster, 1959). More direct evidence for participation of gaseous oxygen in methane oxidation came in 1970 when $^{18}\text{O}_2$ was shown to be incorporated into methanol during methane oxidation in whole-cell suspensions of Methylobionas methanica and Methanomonas methano-oxidans. There was negligible incorporation of oxygen label from water into methanol, which rules out a hydroxylase reaction (1), where water provides the oxygen atom for insertion into methane (Higgins and Quayle, 1970).



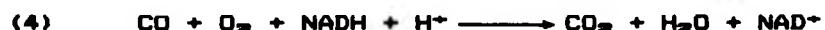
Analogy with the oxidation of higher alkanes and aromatics (see 1.3.A., Table 2) implicated either a monooxygenase reaction (2) or a dioxygenase reaction (3) in the initial methane oxidation steps:-





In the monooxygenase reaction (2) one atom of molecular oxygen is inserted into the substrate and the other atom of oxygen is reduced to water, using reducing equivalents supplied by the oxidation of XH_2 , whereas for a dioxygenase reaction (3) both atoms of molecular oxygen are incorporated into substrate(s) (see Hayaishi, 1974 for review).

The first report of a methane-oxidizing cell-free extract was in 1970 (Ribbons and Michalover), when a particulate preparation of Methylococcus capsulatus (Texas) showed methane stimulated oxygen and NADH consumption, tentatively in equimolar amounts, which is consistent with the stoichiometry of a monooxygenase reaction. However neither methane disappearance nor product accumulation was demonstrated in this system until 1975 (Ribbons). A similar stoichiometry was found for cell-free particulate extracts from Methylomonas methanica (Ferenci, 1974), although with carbon monoxide as a substrate, the stoichiometry for the overall reaction was shown to be consistent with a monooxygenase reaction (Ferenci et al., 1975):-



Using the methane analogue bromomethane, Colby et al. (1975) demonstrated the oxygen and NAD(P)H-dependent disappearance of substrate with the particulate extract from Methylomonas methanica, and the stoichiometric relationship between NADH disappearance and bromomethane disappearance was consistent

with a monooxygenase reaction.

The first report of methanol accumulation from methane in a cell-free system was in 1975 with particulate extracts of Methylosinus trichosporium (OB3b), using a high level of phosphate (150 mM) (Higgins and Quayle, 1970) to inhibit methanol-oxidase activity, allowing methanol to accumulate (Tonge et al., 1975). Although this was the first clear evidence that methanol was the initial product of methane oxidation, the stoichiometric relationship between oxygen consumption and product formation (1.3:0.9) was inconsistent with a monooxygenase reaction for methane oxidation. Subsequently it was shown that high phosphate was not a reliable method for inhibiting methanol-oxidase activity, offering an explanation of this stoichiometry (H.Dalton, personal communication).

In summary, there appears to be direct evidence that a methane oxygenase reaction is operating in methane-oxidizers in general, and stoichiometric evidence which is consistent with a methane monooxygenase reaction being operative. Direct evidence for a monooxygenase reaction might be provided by using a purified methane oxygenase to investigate partitioning of $^{18}\text{O}_2$ label into water and methanol, together with the stoichiometry of electron donor consumption. Unfortunately, no stoichiometric information is available for the soluble methane oxygenase of Methylococcus capsulatus (Bath); though the ability of this enzyme system in crude extracts to catalyze NADH and oxygen-dependent methane oxidation to methanol, in the presence of cyanide to inhibit methanol-oxidase activities, is consistent with a monooxygenase reaction (Colby and Dalton, 1976).

1.2.B. The nature of the reducing agent in the methane oxidation reaction

Methylosinus trichosporium OB3b and Methylococcus capsulatus (Bath) have been shown to possess both particulate and soluble methane monooxygenase activities (Tonge et al., 1975, 1977; Stirling and Dalton, 1979; Stanley et al., 1983; Colby and Dalton, 1976). Most cell-free methane monooxygenase activities from a variety of methane oxidizers, including Methylococcus capsulatus (Bath) and the soluble enzyme activity of Methylosinus trichosporium OB3b appear to have an obligatory requirement for NAD(P)H for in vitro methane monooxygenase activity (Colby and Dalton, 1976; Stirling and Dalton, 1979; see Higgins et al., 1981 for review). The methane monooxygenases are therefore referred to as "external" monooxygenases, inasmuch as reducing equivalents are not supplied by methane but by an external reducing agent. The requirement for NAD(P)H as a reducing agent appears to be typical of the alkane and aromatic oxygenases in general (see 1.3.A.).

There are several reports that ascorbate can act as an electron donor for cell-free methane monooxygenase activity (Hou et al., 1982; Tonge et al., 1975; Pilayashenko-Novokhatnyi et al., 1979). For example in cell-free particulate extracts from Methylosinus trichosporium OB3b ascorbate, methanol and NAD(P)H were able to support methane monooxygenase activity (Tonge et al., 1975); although there has been some difficulty in repeating this work (Stirling and Dalton, 1979; Scott et al., 1981).

The inhibitor profiles of cell-free methane monooxygenases appear to correlate with the location of the enzyme. Particulate extracts of Methylosinus trichosporium QB3b are sensitive to chelating agents, thiol reagents and electron transport inhibitors, suggesting that the enzyme retains a close association with membrane-bound electron transfer proteins and that NAD(P)H may only be an indirect electron donor in vivo (Tonge et al., 1977; Scott et al., 1981). Similar inhibitor sensitivities have been reported for the particulate cell-free extracts from Methylococcus capsulatus (Texas) (Ribbons, 1975), Methylomonas methanica (Ferenci, 1974; Colby et al., 1975), and for Methylosinus sp. CRL-15 (Patel et al., 1979). The soluble activity is insensitive to these inhibitors, suggesting that NAD(P)H interacts directly with this soluble enzyme (Stirling and Dalton, 1979; Scott et al., 1981). A similar situation appears to hold for particulate and soluble cell-free extracts from Methylobacterium organophilum CRL-26 (Patel et al., 1982), and for Methylococcus capsulatus (Bath) where the NAD(P)H-dependent particulate methane monooxygenase is also sensitive to chelating agents, thiol agents and electron transport inhibitors (Stanley et al., 1983). However, in vivo, compounds such as ethanol which are incapable of yielding NAD(P)H directly, can supply reducing equivalents to the particulate methane monooxygenase, possibly by reversed electron transport, again suggesting an indirect role for NAD(P)H as electron donor (Leak and Dalton, 1983). The NAD(P)H-dependent soluble methane monooxygenase is insensitive to these inhibitors (Stirling

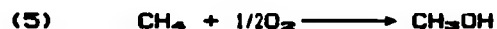
and Dalton, 1977), and appears to be obligatorily NAD(P)H-linked since no other likely electron donors, including ascorbate, supported enzyme activity (Colby and Dalton, 1976; Colby et al., 1977).

The location of the methane monooxygenase in Methylococcus capsulatus (Bath) and Methylosinus trichosporium DB3b appears to be dependent upon the availability of copper. Under conditions of copper stress, the soluble enzyme is present whereas under conditions of copper excess the particulate enzyme predominates. However, this copper-dictated effect on methane monooxygenase location was not found in Methylomonas albus BGG where there was evidence for particulate activity under all growth conditions, so this copper effect is not applicable to all methane-oxidizers (Stanley et al., 1983).

1.3. The mechanism of the methane oxidation reaction

1.3.A. Electron transport pathways in the methane monooxygenase

Since the free-energy change for the methane to methanol oxidation reaction (5) is -26 kcal/mole (Anthony, 1982), indicating that the reaction is essentially irreversible, why is NAD(P)H required?



The most likely reason is that the reducing equivalents from NAD(P)H are used by the methane monooxygenase to surmount a kinetic barrier. This barrier

is probably the very large potential barrier to the activation of oxygen (see Hayaishi, 1974 for review). Thus the inactive nature of biradical molecular oxygen is the reason why reaction (5) does not occur spontaneously at room-temperature. Inspection of the methane monooxygenase equation (6) indicates that the reducing equivalents from NAD(P)H end up in water, probably complexed with an oxygen atom derived from dioxygen, reaction (7). The free-energy change for reaction (7) is -53 kcal/mole.



The most likely explanation for the use of this reducing power is to produce "activated oxygen" (see Hamilton, 1974 for review), capable of (net) insertion of an oxygen atom into a C-H bond of methane to give methanol. A priori, water could be produced either before, concomitantly with, or after the oxygen insertion step. For the P-450 monooxygenase system the first choice has been speculated (Coon and White, 1980).

The soluble methane monooxygenase of Methylococcus capsulatus (Bath) is a multi-component enzyme system (Colby and Dalton, 1976, 1978), as are the other two methane monooxygenases which have been investigated (Tonge et al., 1977; Patel et al., 1982) and many other alkane and aromatic oxygenases (Table 2). These multi-component oxygenases may

Table 2. Electron transport chains in some multi-component alkane and aromatic oxygenases.

| System | Donor | Components | | |
|--|-----------|---------------------------------------|-----------------------------------|---|
| Dioxygenases | | | | |
| Benzene dioxygenase ^A <u>P. putida</u> | NADH | (FAD) | (Fe ₂ S ₂) | (2Fe ₂ S ₂) + Fe ²⁺ |
| Toluene dioxygenase ^B <u>P. putida</u> | NADH | (FP) | (Fe ₂ S ₂) | (Fe ₂ S ₂ ...Fe) |
| Pyrazon dioxygenase ^C pyrazon degrading <u>sp.</u> | NADH | (FAD) | (Fe ₂ S ₂) | (Fe ₂ S ₂) |
| Benzoate dioxygenase ^D <u>P. arvilla</u> | NADH | (FAD Fe ₂ S ₂) | | (Fe ₂ S ₂ ...Fe) |
| Monooxygenases | | | | |
| Putidamonooxin ^E <u>P. putida</u> | NADH | (FMN FeS) | | (Fe ₂ S ₂ ...Fe) |
| Alkane hydroxylase ^F <u>P. oleovorans</u> | NADH | (FAD) | (2Fe) | (2Fe ³⁺) |
| Alkane hydroxylase ^G <u>Corynebacterium sp.</u> | NADH | (FP) | | (P-450) |
| Camphor hydroxylase ^H <u>P. putida</u> | NADH | (FAD) | (Fe ₂ S ₂) | (P-450) |
| P-450 monooxygenase ^I microsomal | NADPH | (FAD FMN) | | (P-450) |
| P-450 monooxygenase ^J mitochondrial | NADPH | (FAD) | (Fe ₂ S ₂) | (P-450) |
| Methane monooxygenase ^K <u>M. capsulatus</u> (Bath) | NAD(P)H | (FAD Fe ₂ S ₂) | () | (Fe...Fe) |
| Methane monooxygenase ^L <u>M. trichosporium</u> OB3b | Ascorbate | (cyt.c) | () | (Cu) |

References to Table 2.

- A (Axcell and Geary, 1975; Crutcher and Geary, 1979;
Geary and Dixon, 1981)
- B (Yeh et al., 1977; Subramanian et al., 1979)
- C (Sauber et al., 1977)
- D (Yamaguchi and Fujisawa, 1978, 1982)
- E (Bernhardt et al., 1975; Bill et al., 1981)
- F (McKenna and Coon, 1970; Ruettiger et al., 1977)
- G (Cardini and Jurtshuk, 1970)
- H (Katigiri et al., 1968; Peterson and Mock, 1975)
- I (Lu et al., 1969; Iyanagi and Mason, 1973)
- J (Omura et al., 1966; Baron et al., 1972)
- K (Colby and Dalton, 1978; Woodland and Dalton, 1983)
- L (Tonge et al., 1975, 1977)

be resolved into separate components and reconstituted for oxygenase activity. In common with most oxygenases, the alkane and aromatic oxygenases use external donors for reducing equivalents, usually reduced pyridine nucleotides, mainly NADH. The exception to the oxygenases chosen here is the methane monooxygenase of Methylosinus trichosporium OB3b, which is reported to use ascorbate as a donor of reducing equivalents (Tonge et al., 1977). For the soluble methane monooxygenase of Methylococcus capsulatus (Bath) NADPH supports monooxygenase enzyme activity at roughly 50% of the rate seen with NADH, at a concentration of 5mM (Colby and Dalton, 1976).

The components of these oxygenase systems appear to have discrete functions. One or more of the components

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- A (Axcell and Geary, 1975; Crutcher and Geary, 1979;
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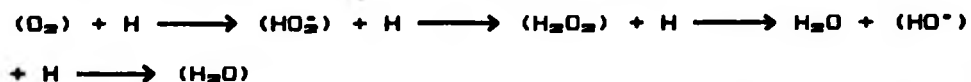
forms a short electron transport chain from NAD(P)H to the oxygenase component, usually via two redox centres. The initial acceptor of reducing equivalents usually appears to be a flavoprotein, often possessing independent reductase activities, with either an FAD or an FMN as a prosthetic group, both of which can accept two electrons. However the methane monooxygenase of Methylosinus trichosporium OB3b appears to use an iron containing cytochrome c as the initial electron acceptor (Tonge et al., 1977), which might be expected to accept only one electron at a time. Reducing equivalents are then transferred to a second redox centre, often an FMN or an Fe_2S_2 redox centre, which appears to accept only one electron, and transfers one electron at a time to the redox centre(s) of the oxygenase component. The first two redox centres in this chain may be together on one component, as is the case for the methane monooxygenase of Methylococcus capsulatus (Bath) (Colby and Dalton, 1978, 1979), or separate on two components. The oxygenase component usually contains some form of iron, either a P-450 centre, an iron-sulphur centre, an iron centre, or iron complexed with an iron-sulphur centre. The putative oxygenase component of the Methylosinus trichosporium OB3b particulate enzyme system contains copper (Tonge et al., 1977).

The role of these short electron transport chains may be to split up high potential electron pairs from NAD(P)H and to transfer them to the oxygenase in one electron steps at a constant redox potential (see Kamin et al., 1980; Kamin and Lambeth, 1982 for reviews of this type

of electron transport chain). Electron transport chains of this nature are not restricted to oxygenases, and such a chain operates for the E. coli sulphite reductase, for example (Siegel et al., 1974; Siegel and Davis, 1974). The corollary to employing an intermediate one electron transfer redox centre is that electrons may only be required one at a time, for instance in the P-450 system it has been proposed that in the presence of substrate one electron is transferred to the monooxygenase component to give ferrous iron, which then binds dioxygen, followed by a second electron (see Coon and White, 1980 for review).

1.3.B. The oxygen activation step in the methane monooxygenase reaction

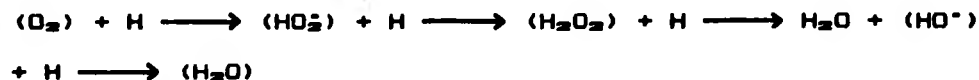
Molecular oxygen is chemically unreactive at room temperature, and is thought to be inert because it is a biradical, a triplet spin-system. Direct reaction with singlet hydrocarbons to give singlet products is spin forbidden due to the requirement to conserve the spin angular momentum in a chemical reaction. Therefore, although such a reaction would be exergonic it is kinetically controlled (Hamilton, 1974). There are two ways in which this activation could be achieved; one is by oxidation of the oxygen molecule to ozone, but biological systems do not produce ozone so this method has been discounted (Williams, 1980); the alternative is reduction, for which four reducing equivalents are required to effect full reduction of a molecule of oxygen:-



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1.3.B. The oxygen activation step in the methane monooxygenase reaction

Molecular oxygen is chemically unreactive at room temperature, and is thought to be inert because it is a biradical, a triplet spin-system. Direct reaction with singlet hydrocarbons to give singlet products is spin forbidden due to the requirement to conserve the spin angular momentum in a chemical reaction. Therefore, although such a reaction would be exergonic it is kinetically controlled (Hamilton, 1974). There are two ways in which this activation could be achieved; one is by oxidation of the oxygen molecule to ozone, but biological systems do not produce ozone so this method has been discounted (Williams, 1980); the alternative is reduction, for which four reducing equivalents are required to effect full reduction of a molecule of oxygen:-



Catalase and superoxide dismutase have no effect on oxidations mediated by Methylococcus capsulatus (Bath), indicating that free peroxide or superoxide does not act as the oxygenating agent (Colby et al., 1977). It is unlikely that a hydroxyl radical is used, because it is so reactive that it would not produce the specificities shown by these enzymes, and would probably react with the enzyme itself. The most likely oxidation level would be that of the peroxide, by analogy with, for instance, the peracid epoxidation of alkenes (Hamilton, 1974). Protein A, the oxygenase component of the three component soluble methane monooxygenase from Methylococcus capsulatus (Bath), has an EPR-active iron prosthetic group, so some sort of oxygen-iron complex could be a possibility (Woodland and Dalton, 1983). A common feature of many oxygenases is that only in the presence of substrate does oxygen bind and become activated (see Hayaishi, 1974 for review). With anaerobic semi-reduced protein A, substrates such as cyanomethane and ethene appear to enhance the EPR signal putatively assigned to a spin-coupled iron centre, indicating that the substrate may be able to bind to protein A in the absence of oxygen, which would merely be consistent with the above generalization (Colby et al., 1979; Woodland and Dalton, 1983; M.P.Woodland, personal communication, 1983).

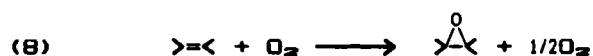
Evidence that the "activated oxygen" in the methane monooxygenase of Methylococcus capsulatus (Bath) appears to be electrophilic comes from studies with

alternate substrates (see 1.3.C). Oxidation of mono-substituted benzenes produces mainly para-substituted phenols, as opposed to meta-substituted phenols, which is consistent with an attack by an electrophilic oxygen species (Dalton et al., 1981; Waters, 1982). Spin-trapping experiments with Methylosinus trichosporium DB3b have failed to detect radicals, which would be consistent with activation of oxygen not proceeding through a radical mechanism (see Higgins et al., 1981).

By analogy with proposals for the P-450 system, which also produces "activated oxygen" capable of attacking an alkane at a C-H bond which has not been activated, the immediate precursor of "activated oxygen" could be produced either by heterolytic cleavage of the peroxide bond, which together with proton uptake would lead to the extrusion of water, or by homolytic cleavage of the peroxide bond with extrusion of water (Coon and White, 1980). The two reducing equivalents from NAD(P)H would then end up in this water molecule with one atom of dioxygen and the other atom of dioxygen would be present as some form of ferric iron-oxygen complex. The reducing power from the NAD(P)H would then have been used in order to produce "active oxygen"; singlet oxygen which is electron deficient and therefore electrophilic. However the "active oxygen" species in the P-450 system may be different to that in the methane monooxygenase system of Methylococcus capsulatus (Bath) because chlorite and periodate will not replace the requirement for reducing power and oxygen for substrate oxidation by the methane monooxygenase (Colby et al., 1977),

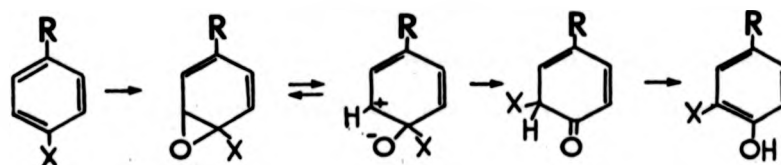
whereas replacement is seen for the P-450 system (Hrycay et al., 1975; see White and Coon, 1980 for review).

Evidence for the methane monooxygenase of Methylococcus capsulatus (Bath) exhibiting an electron deficient singlet oxygen atom comes from studies carried out with alkenes, when epoxides are formed by an addition reaction. For example with ethene, epoxyethane is produced (8) (Colby et al., 1977). This is analogous to the carbene reaction with alkenes to give cyclopropanes (9) (Hamilton, 1974).



Epoxidation of arenes has also been shown to occur with the soluble methane monooxygenase of Methylococcus capsulatus (Bath) (Dalton et al., 1981). The epoxide itself is unstable and reacts to give a ring hydroxylation by a mechanism called the "NIH shift", which has been found for the P-450 system (see Coon and White, 1980 for review), for aromatic hydroxylases (see Hamilton, 1974 for review), and for the Methylococcus capsulatus (Bath) system during ring oxidation of 1-phenylethane (Dalton et al., 1981), see Figure 2.

Figure 2. The mechanism of the "NIH" shift.



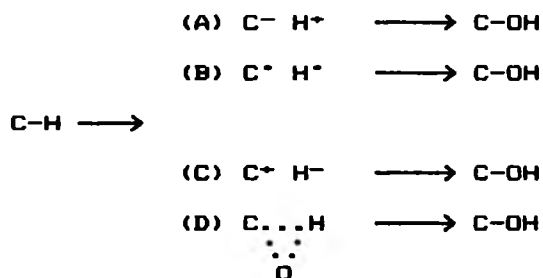
The epoxide is in equilibrium with a ring opened zwitterion which decays to a dienone with, for example, 1,2-deuterium migration, and a second switch occurs on tautomerization to the substituted phenol.

1.3.C. The mechanism of substrate oxidation in the methane monooxygenase reaction

Cell-free extracts of the soluble methane monooxygenase of Methylococcus capsulatus (Bath) appear to be able to oxidize a wide range of hydrocarbon substrates, including substituted methane derivatives, amines, alkanes, alkenes, ethers, alicyclics, heterocyclics and aromatics (Colby et al., 1977; Dalton, 1977; Stirling and Dalton, 1979). The soluble methane monooxygenase of Methylosinus trichosporium OB3b appears to have a similar broad substrate range in cell-free extracts (Stirling et al., 1979), like the soluble methane monooxygenase of Methylobacterium organophilum CRL-26 (Patel et al., 1982), but unlike the particulate cell-free extract of Methylomonas methanica, which does not oxidize n-alkanes with more than six carbon atoms, or alicyclic, heterocyclic and aromatic compounds (Stirling et al., 1979). This restricted substrate range may reflect the difference in the location of this particulate methane monooxygenase, as compared to the three soluble monooxygenases investigated. The possession of a broad substrate range allows mechanistic insights to be gained, by investigating the products of different substrates. A priori, insertion of "activated oxygen" into carbon-hydrogen bonds or addition to carbon-carbon double

bonds could proceed by any of four mechanisms (Coon and White, 1980; see Figure 3), namely initial abstraction of a proton (A), a hydrogen atom (B) or a hydride ion (C) to leave a carbanion, a radical or a carbonium ion, followed by subsequent insertion of the "activated oxygen"; or via a concerted insertion mechanism (D), termed the "oxenoid" mechanism (Hamilton, 1964).

Figure 3. Possible mechanisms of oxygen insertion by monooxygenase enzymes.



The "oxenoid" mechanism was proposed by analogy to carbene reactions (Hamilton, 1964). Like the putative oxygen atom, carbenes and nitrenes possess six electrons in their outer shell, and are electrophilic. Retention of configuration is observed on insertion of nitrenes and carbenes into alkanes, as would be predicted for a concerted insertion mechanism. For the other mechanisms the degree of retention of configuration would be determined by the stabilities of the intermediates produced, namely the carbanion, the radical, or the carbonium ion.

With soluble cell-free extracts from Methylococcus

capsulatus (Bath) oxidation of cis-but-2-ene and trans-but-2-ene proceeds with retention of configuration (Colby et al., 1977), and neither is inversion seen with cis-[1-²H]propene (Waters, 1982). This is consistent with an "oxenoid" mechanism, at least for addition to alkenes. Recent available evidence tends to mediate against the participation of a hydrocarbon radical in the oxygen insertion step of the methane monooxygenases, although earlier evidence appeared to be consistent with such a mechanism (Hutchinson et al., 1976). For a hydrocarbon radical mechanism oxidation of small aliphatics might be expected to result in a preference for tertiary positions over secondary positions, with primary positions disfavoured, as occurs for aliphatic hydroxylations by the P-450 system (White et al., 1979). In the P-450 system alkane hydroxylation may not proceed with retention of configuration and an intramolecular deuterium isotope effect is seen in hydrogen/deuterium competition experiments (Groves et al., 1978). These data are consistent with a selective hydrogen abstraction, but is not consistent with an "oxenoid" mechanism. However, this preference does not appear to hold for oxidations with the monooxygenase from Methylococcus capsulatus (Bath) (Colby et al., 1977), the Methylosinus trichosporium OB3b soluble enzyme system (Stirling et al., 1979), nor with the soluble methane monooxygenase from Methylobacterium organophilum CRL-26 (Patel et al., 1982). The "oxenoid" and carbanion mechanisms would predict little selectivity for oxygen insertion into primary, secondary, or tertiary aliphatics,

and are therefore consistent with this observation. The assumption implicit in these predictions of selectivity is that the oxygen insertion step is at least partially rate-determining for the overall monooxygenase reaction. Similarly, studies with the alicyclic methylcyclopropane also tend to preclude a radical mechanism for oxygen insertion, since no ring-opened products are seen. A methylcyclopropyl radical would be expected to cleave rapidly and spontaneously to the 3-butenyl radical giving ring-opened products. With cyclopropane, allyl alcohol is not formed, which empirically indicates against a carbocation mechanism (Dalton et al., 1981). With Methylosinus trichosporium OB3b spin-trapping experiments have not shown up any radical participation (see Higgins et al., 1981), so substrate activation in the methane monooxygenases may not proceed generally through a radical mechanism, in contrast with the P-450 system.

However, oxidation of substituent groups on the aromatic ring occurs almost exclusively alpha to the aromatic ring, which would be expected if a radical or charged intermediate were produced enabling stabilization by the adjacent aromatic ring. Therefore a carbanion mechanism, due to proton abstraction, is not ruled out, at least for aromatics. But if this stabilization is a strict requirement, then it is hard to see why aliphatic compounds like methane are oxidized (Waters, 1982). In summary, the available evidence tends to favour methane oxidation proceeding by a concerted insertion of "activated oxygen" into a carbon-hydrogen bond.

1.4. The purification and characterization of the methane monooxygenases

The majority of the methane monooxygenases are particulate and are also very unstable in cell-free extracts (see Dalton, 1980 (b), for review). This instability, combined with the difficulties of solubilizing these enzyme systems from the membrane in active form, has prevented purification of all but a few of these enzyme systems.

1.4.A. The multi-component nature of the methane monooxygenases

All of the methane monooxygenases amenable to investigation have been shown to be multi-component enzyme systems. Resolution of the components catalyzing methane monooxygenase activity has been demonstrated for three methane-oxidizing bacteria (Table 3).

The first report of the resolution of a methane monooxygenase was for the particulate enzyme system of Methylosinus trichosporium OB3b (Tonge et al., 1975). The particulate component(s) were solubilized by means of phospholipase-D, and gave activity when this fraction was combined with a soluble CO-binding cytochrome c. Subsequently three soluble methane monooxygenases were resolved into two or three fractions by ion-exchange chromatography and appeared to have several similarities. The Methylobacterium organophilum CRL-26 fractions were resolved on DEAE-cellulose in an identical manner to the Methylococcus capsulatus (Bath) fractions and for both enzyme systems the fraction A (eluted with 0 M NaCl) and

Table 3. The resolved methane monooxygenase activities.

| Bacterium | enzyme localization | fractions |
|-------------------------------|---------------------|-------------------------|
| <u>M. organophilum</u> CRL-26 | soluble | DEAE:A,B,C |
| <u>M. capsulatus</u> (Bath) | soluble | DEAE:A,B,C |
| <u>M. trichosporium</u> OB3b | soluble | DEAE:1,2 |
| <u>M. trichosporium</u> OB3b | particulate | 1,2,cyt c _{oo} |

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- A (Patel et al., 1982)
- B (Colby and Dalton, 1976, 1978)
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- B (Colby and Dalton, 1976, 1978)
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fraction C (eluted with 0.5 M NaCl) were absolute requirements for enzyme activity, whereas fraction B (0.2M NaCl) simply stimulated activity approximately two-fold (Colby and Dalton, 1978; Patel et al., 1982). Moreover, the fractions B and C together of Methylococcus capsulatus (Bath) could substitute for the fraction 2 (0.5M NaCl) of Methylosinus trichosporium DB3b in reconstituting methane monooxygenase activity with fraction 1 (0M NaCl), indicating a strong functional analogy between these two soluble systems, and that fraction 1 may contain the monooxygenase component (Stirling and Dalton, 1979). In summary, the methane monooxygenases appear generally to be multi-component enzyme systems.

1.4.B. The stability of the methane monooxygenases

Stability data is available for three of the four resolved methane monooxygenases, namely the soluble enzyme from Methylococcus capsulatus (Bath) and the soluble and particulate enzymes from Methylosinus trichosporium DB3b methane monooxygenases in cell-free extracts. Cell-free soluble extracts from Methylococcus capsulatus (Bath) retained 75-100% of their activity at 0°C for 24 hours and were completely stable for several months at -70°C (Colby and Dalton, 1976). The instability of these preparations was found to be due to the decay of component C, present in the resolved DEAE-fraction C. DEAE-fraction C lost 60-90% of its activity at 0°C for 20 hours, whereas DEAE-fractions A and B retained full activity at 0°C for 24 hours, although DEAE-fraction A lost up to 40% of its activity at 4°C for 72

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Stability data is available for three of the four resolved methane monooxygenases, namely the soluble enzyme from Methylococcus capsulatus (Bath) and the soluble and particulate enzymes from Methylosinus trichosporium OB3b methane monooxygenases in cell-free extracts. Cell-free soluble extracts from Methylococcus capsulatus (Bath) retained 75-100% of their activity at 0°C for 24 hours and were completely stable for several months at -70°C (Colby and Dalton, 1976). The instability of these preparations was found to be due to the decay of component C, present in the resolved DEAE-fraction C. DEAE-fraction C lost 60-90% of its activity at 0°C for 20 hours, whereas DEAE-fractions A and B retained full activity at 0°C for 24 hours, although DEAE-fraction A lost up to 40% of its activity at 4°C for 72

hours (Woodland and Dalton, 1983). Fraction C was found to be stabilized by 5mM sodium thioglycollate (Colby and Dalton, 1978). Later DEAE-fraction B was reported to be unstable above 0°C, unless phenylmethanesulphonylfluoride, a protease inhibitor, was present. All three DEAE-fractions were reported to be unstable above 0°C, although all the DEAE-fractions were stable for at least three months if kept frozen below -20°C (Colby et al., 1979).

The particulate methane monooxygenase of Methylosinus trichosporium OB3b was found to be stable in cell-free crude extracts, losing no activity at 4°C for 14 days. The particulate fraction was also stable, provided that the soluble cytochrome c_{oo} was present, losing no activity at 4°C for 7 days (Tonge et al., 1975). All three purified components of the particulate methane monooxygenase were found to be stable at 0-4°C. The two solubilized and purified particulate proteins lost only 4% of their activity at 0°C for 3 weeks, and 30% of their activity was lost on storage at 4°C for two weeks. The purified soluble CO-binding cytochrome c was stable at 0-4°C for at least 6 months. All three components were inactivated by one freeze-thaw cycle (Tonge et al., 1977). Later, apparently contradictory results were reported with respect to stability. Particulate extracts were found to be stable when frozen and stored under liquid nitrogen, but unstable at 0-4°C, losing all activity after storage overnight (Scott et al., 1981). However, these authors do not report supplementing the particulate extracts with the soluble cytochrome c_{oo}, which was indicated as a stabilizing agent

for particulate extracts. In addition, the previous specific activities of the particulate extract were an order of magnitude larger than those reported by Scott et al. (1981), in fact the recent activity was at a low level which was very similar to the specific activity found for particulate extracts in the absence of the soluble CO-binding cytochrome c (Tonge et al., 1975). Scott et al. (1981) report that the cytochrome did not enhance methane monooxygenase activity as indicated by Tonge et al. (1975, 1977). But these later workers used frozen purified cytochrome c_{oo}, which was earlier reported to be inactivated by freezing (Tonge et al., 1977), and therefore do not appear to have formally refuted the earlier results.

Cell-free crude extracts of the soluble methane monooxygenase of Methylosinus trichosporium OB3b were found to lose all activity on storage at 4°C overnight but were stable to freezing and storage at -80°C, unlike soluble extracts, which retained only 25% of their activity on storage at -80°C for 10 days. Soluble extracts lost all activity in 2 hours at 4°C, as did DEAE-fraction 1 at 4°C. The latter fraction was also unstable at -80°C. The component(s) in DEAE-fraction 1 showed more stability in soluble and crude cell-free extracts, as activity could be complemented after several hours at 4°C with DEAE-fractions B and C from the soluble methane monooxygenase of Methylococcus capsulatus (Bath) (Stirling and Dalton, 1979).

Later, the instability of the soluble enzyme, and its instability in soluble extracts was confirmed. The activity was partially stabilized with either

phenylmethylsulphonylflouride (contrary to the report of Stirling and Dalton, 1979) or by anaerobic conditions, so that 40-45% of the activity was retained at 0-4°C for 24 hours (Scott et al., 1981).

In summary, the soluble methane monooxygenases from Methylococcus capsulatus (Bath) and Methylosinus trichosporium OB3b appear to be reasonably stable, in the presence of appropriate stabilizing agents, whereas the stability of the particulate enzyme from Methylosinus trichosporium OB3b is at present equivocal, but may be excellent.

1.4.C. The purification and characterization of two methane monooxygenases

Active methane monooxygenase components appear to have been purified and characterized in two cases; the particulate methane monooxygenase of Methylosinus trichosporium OB3b (1) (Tonge et al., 1975, 1977), and the soluble methane monooxygenase of Methylococcus capsulatus (Bath) (2) (Colby and Dalton, 1978, 1979; Dalton, 1980).

(1) The particulate methane monooxygenase from Methylosinus trichosporium OB3b was solubilized from the membrane by using phospholipase-D. A soluble CO-binding cytochrome c was necessary for high level activity with this solubilized fraction and with the cell-free particulate fraction. The soluble preparation had no NADH/ascorbate oxidase activity, unlike the soluble cytochrome c_{550} . In crude and in particulate extracts supplemented with the cytochrome c_{550} , ascorbate (1.5mM) could act as an alternate

electron donor to NADH, as could methanol in crude extracts (Tonge et al., 1975). The cytochrome c_{550} was purified by precipitation of nucleoproteins from soluble crude extract using protamine sulphate, followed by DEAE-cellulose treatment. The non-binding supernatant was subjected to Sephadex G-150 and Sephadex G-50 gel-permeation column chromatography followed by hydroxyapatite column chromatography (Tonge et al., 1977). The purified cytochrome c_{550} had a molecular weight of 13,000, 1 iron atom and 0.3-0.8 copper atoms per polypeptide. Because of its endogenous oxidase activities, the cytochrome c_{550} was proposed to be the reductase component of the methane monooxygenase, i.e. the immediate electron donor for the monooxygenase component, but due to its ability to bind substrates such as oxygen and carbon monoxide, and later also methane and ethane (Hammond et al., 1979), the possibility of the cytochrome c_{550} being the monooxygenase component was not ruled out (Tonge et al., 1977). However, later it was found that the cytochrome c_{550} was not required for particulate methane monooxygenase activity in cell-free particulate extracts, albeit at a specific activity ten-fold reduced compared with that of the previous workers, who had already shown that this level of activity was attainable without the supplementary cytochrome c_{550} (Tonge et al., 1975). In the later study only NAD(P)H could act as an electron donor to the particulate methane monooxygenase, neither ascorbate nor methanol would serve, though occasionally ascorbate/tetramethyl-para-phenylene-diamine gave a low level activity (Scott et al., 1981).

The solubilized particulate fraction was resolved into two further fractions by ultrafiltration with a PM10 membrane. Protein 1 was purified from the supernatant, fraction 1, by Sephadex G-200 and Sephadex G-75 column chromatography with a 66% yield, and had a molecular weight of 47,000 and 1 copper atom per polypeptide chain. Protein 2 was purified from the PM10 eluate, fraction 2, by Sephadex G-25 and Sephadex G-50 column chromatography, and had a molecular weight of 9,400. Due to the possession of copper, protein 1 was proposed to be the monooxygenase component. Maximal activity was found with a 1:1:1 protein stoichiometry for the three components, with a specific activity of approximately 6 micromoles/minute/mg protein. The purified enzyme system could use only ascorbate as electron donor and was not active with NAD(P)H. Activity with NAD(P)H was reported lost on Sephadex G-200 chromatography of fraction 1, and the loss was thought to be due to removal of electron transport components from protein 1. However, methanol could drive the purified oxygenase system in the presence of partially purified methanol dehydrogenase. No definite conclusions were drawn about the functions of the various components (Tonge *et al.*, 1977). Unfortunately, Higgins (1981) reports that the purification procedure employed previously was no longer producing active enzyme in their laboratory, so no more details of this fascinating particulate system have become available.

Figure 4. The putative pathway of electron flow in the particulate methane monooxygenase of *M. trichosporium* OB3b.



* No function has been ascribed to protein 2.

(2) Soluble extracts of the methane monooxygenase from *Methylococcus capsulatus* (Bath) were initially resolved into two fractions by DEAE-cellulose column chromatography (Colby and Dalton, 1976), and then into three fractions by the same technique (Colby and Dalton, 1978). On ion-exchange, fraction A did not bind to the DEAE-cellulose column, fraction B was eluted with 0.2M NaCl and fraction C with 0.5M NaCl. DEAE-fractions A and C were absolute requirements for methane monooxygenase activity, although DEAE-fraction B was found to stimulate activity between 2-fold and 4-fold, and was shown not to possess any fraction A or fraction C activity. None of the fractions had any activity on their own. The only combination of two fractions with activity was the combination of fraction A with fraction C (Table 4).

Table 4. Reconstitution of methane monooxygenase activity*
from DEAE-fractions A, B, and C.

| Additions (mg protein) | ethylene oxide formed (micromoles/minute) |
|---------------------------|--|
| A(3.4), B(1.3), C(0.3) | 0.28 |
| A(3.4), B(1.3) | 0 |
| A(3.4), B(3.9) | 0 |
| A(3.4), C(0.3) | 0.07 |
| B(1.3), C(0.3) | 0 |
| B(3.9), C(0.3) | 0 |

* Methane monooxygenase activity was determined by measuring epoxyethane formation from ethene over a 10 minute period and reactions were initiated by adding the various fractions in the amounts indicated (Colby and Dalton, 1978).

These DEAE-fractions have been studied further and the components responsible for methane monooxygenase activity have been purified and shown to be proteins (Colby and Dalton, 1978, 1979; Dalton, 1980; Woodland and Dalton, 1983). The three DEAE-fractions have each been shown to contain one protein, and all three proteins are essential for in vitro methane monooxygenase activity. The earlier report that DEAE-fraction B simply stimulated the activity of fractions A and C is known now to be due to a small amount of protein B in the DEAE-fraction A preparation

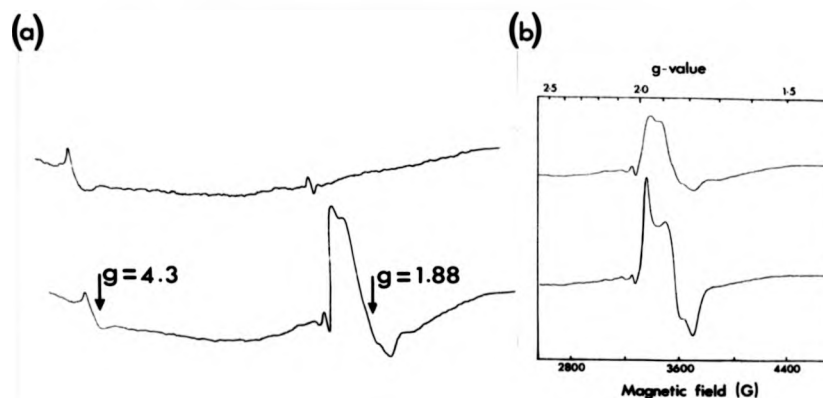
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Figure 7. ESR spectra of protein A.



(a) The upper trace is seen with oxidised protein A, and the lower trace is with protein A reduced by dithionite. The conditions were:- 13 Kelvin, 20 mW microwave power, and a microwave frequency of 9.162 GHz.

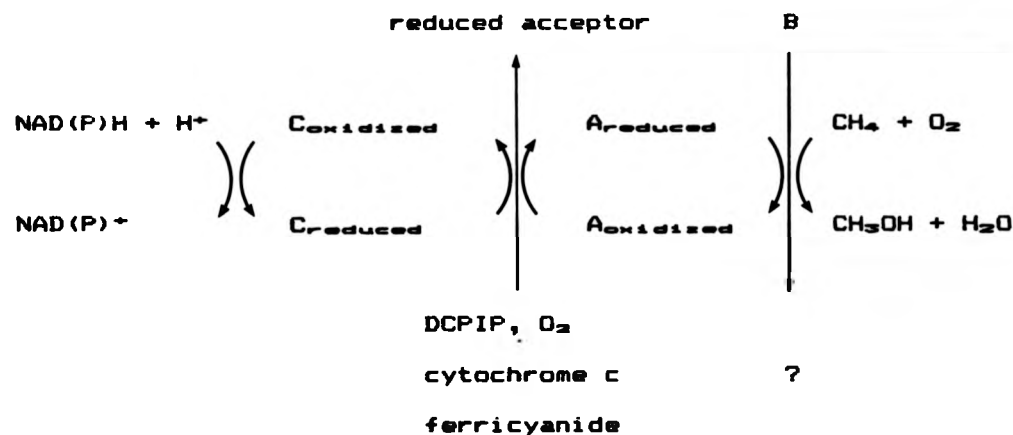
(b) The upper trace is seen with reduced protein A, and the lower trace is of reduced protein A in the presence of 2 mM cyanomethane. The conditions were:- 10 Kelvin, 20 mW microwave power, and a microwave frequency of 9.162 GHz.

oxygenase component of the soluble methane monooxygenase system of Methylococcus capsulatus (Bath) (Colby et al., 1979; Dalton, 1980). Protein B is a colourless protein of polypeptide molecular weight 15,000 by SDS-PAGE (Colby et al., 1979). Like protein A, it has no independent catalytic activities and is an absolute requirement for methane monooxygenase activity, although the role of protein B is unknown (Dalton, 1980).

Protein C is a single polypeptide chain of molecular weight 39,000 by SDS-PAGE and 44,600 by gel permeation chromatography on Sephadex G-100 (Colby and Dalton, 1978). Protein C is yellow-brown in colour, and contains two prosthetic groups, 1 FAD and 1 Fe_2S_2 centre (Colby and Dalton, 1978, 1979). 1 mole of protein C was directly reduced by 1.2 moles of NADH, which was taken to indicate that the protein accepted two electrons for its complete reduction (Colby and Dalton, 1978). Protein C is the only one of the three proteins of the methane monooxygenase to possess independent catalytic activities, namely NAD(P)H-oxidase and NAD(P)H-acceptor reductase activities; when supplied with NAD(P)H protein C can reduce electron acceptors such as oxygen, ferricyanide, cytochrome c or 2,6-dichlorophenolindophenol (DCPIP). The ability of protein C to interact directly with NAD(P)H, the possession of independent reductase activities, and the ability of protein C to reduce protein A under anaerobic conditions, indicate that it has a role in electron transport from NAD(P)H to protein A, the putative monooxygenase component.

Therefore, protein C would appear to be the reductase component of the soluble methane monooxygenase of Methylococcus capsulatus (Bath) (Colby and Dalton, 1978, 1979). Based on these findings, a scheme for electron transfer within the monooxygenase system was proposed, Figure 8 (Dalton, 1980).

Figure 8. The proposed mechanism for electron transfer within the soluble methane monooxygenase complex from M. capsulatus (Bath).

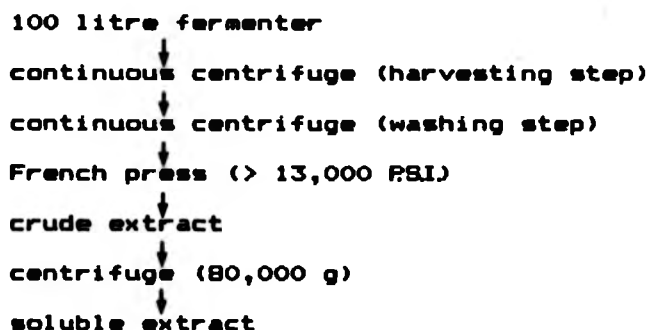


CHAPTER 2. The preparation and characterization of protein C

2.1.A. Growth and harvesting of *Methylococcus capsulatus* (Bath)

Methylococcus capsulatus (Bath) was grown in batch culture in a 100 litre fermenter for 20 hours on methane as the sole carbon and energy source, at 45°C (the optimal growth temperature) and pH 6.8 (4 mM phosphate buffer), supplemented with a nitrate/mineral salts medium (Whittenbury *et al.*, 1970), as detailed in Materials and Methods. The growth and harvesting process, and the method for preparation of soluble extracts (see Materials and Methods, and Figure 2.1.) are that of Colby and Dalton (1976) and Colby *et al.* (1977), with the addition of several minor changes in an attempt to maximize the yield of active methane monooxygenase (MMO) and its specific activity, and both of these properties for protein C in particular (the subject of this thesis).

Figure 2.1. The preparation of soluble extracts of the MMO from *M. capsulatus* (Bath).



(1) The 100 litre fermenter was inoculated with approximately 500 ml of a microscopically clean culture of Methylococcus capsulatus (Bath) from a chemostat. The inoculum used for previous work on this system was 10-20 litres of a continuous culture overflow (Colby and Dalton, 1976, 1978, 1979). This latter method of inoculation is very convenient and quick because the cells are at a high initial O.D., but may not be quite as reproducible on an empirical basis with respect to yielding the large amounts of MMO-active preparations required for successful protein purification, see Table 2.1.

Table 2.1. "Success rate" of 100 litre runs.

| date | 100 litre runs | MMO active | active and O.D. ₆₄₀ >5 | % "success rate" |
|----------------------|----------------|------------|-----------------------------------|------------------|
| 1.2.79- 12.8.80 | 12 | 4-8 | 4-5 | 33-67 |
| 21.1.81- 4.6.81** | 7 | 0 | 0 | 0 |
| 26.6.81- 17.1.83 | 17 | 17 | 16 | 94 |

* I.e. O.D.₆₄₀ > 5, and MMO active, allowing purification of usable amounts of MMO components.

** The cells were grown on ethene-contaminated methane during this period, see 2.1.B.

Doubling times of Methylococcus capsulatus (Bath) in the 100 litre fermenter were variable, usually between 7 and 15 hours, much slower than the initial doubling time seen in the 100 litre fermenter (around 4 hours) and the minimum doubling time reported of around 3.5 hours (Whittenbury et al., 1970), presumably due to the 100 litre fermenter stirring characteristics, and the subsequent rate of oxygen and methane transfer to the cells, which might be expected to be greater in a smaller fermenter with more efficient baffles. For this reason the stirring rate was maintained at a maximum throughout the 100 litre runs.

(2) Oxygen is a substrate of the MMO, but no rigorous study has been made on the effect of oxygen tension on soluble MMO activity, therefore an empirical experiment was carried out to find a satisfactory range of oxygen tensions for growth of Methylococcus capsulatus (Bath), the criteria being reasonable specific activity for MMO activity and reasonable yields of cells. The oxygen tension was detected using a potentiometric oxygen electrode and was regulated by means of an oxygen valve and controller to control the air supply. Oxygen tensions of $pO_2\%$ = 2% and 15% gave soluble preparations in which the specific activity for the MMO and the amount of protein produced were entirely satisfactory; typically the specific activity was circa 0.06 micromole/min/mg for the epoxyethane assay, (as detailed in Materials and Methods), and the mean O.D.₅₄₀ was 11 and gave 1/2-3/4 litre of soluble extract at 80 mg/ml. Therefore an

oxygen tension of 2% was employed for all subsequent 100 litre runs for growth of Methylococcus capsulatus (Bath).

(3) All buffers for harvesting (see Figure 2.1., and Materials and Methods) and subsequent preparation of soluble extract contained 5 mM sodium thioglycollate, a stabilizing agent for protein C (see section 2.3.C.), although previously 5mM sodium thioglycollate was added only at or after the DEAE-cellulose purification step (Colby and Dalton, 1978, 1979; G.Chapman, personal communication).

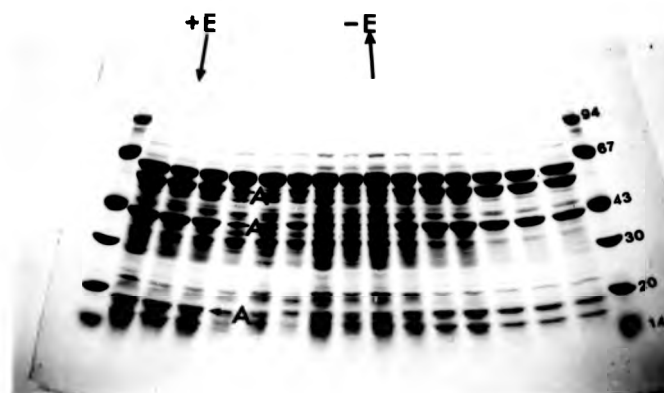
2.1.B. Growth of Methylococcus capsulatus (Bath) on ethene contaminated methane

(1) Inactive soluble extracts

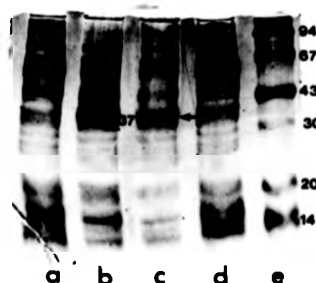
During the period 21.1.81 to 4.6.81 it was only possible to obtain soluble extract with much decreased activity for the MMO (see Table 2.1.), the specific activity varying from 0.001-0.011 micromole/min/mg, as compared to 0.025-0.090 units for normal soluble extracts, and neither was it possible to obtain protein C in anything other than small amounts of semi-pure protein. The cells appeared to grow more slowly in batch culture in a 5 litre fermenter than would be expected for Methylococcus capsulatus (Bath), the maximum doubling time recorded being five hours, as opposed to 3 to 3.5 hours, in a 5 litre fermenter, under conditions where active crude extract is obtained (methane free from ethene contamination), and also gave an extract which was more red in colour than soluble extracts active

for the MMO. These extracts also lacked MMO activity for proteins A, B, and C individually, as well as for the MMO. Protein A polypeptides, normally observable in soluble extracts, comprising 10-30% of the total soluble protein by densitometry of Coomassie stained SDS-PAGE gels, were missing from soluble extracts, see Figure 2.2(a)., (M.P.Woodland, personal communication). Other than the MMO proteins, the SDS-PAGE profiles of the soluble extracts of active and inactive preparations were similar, indicating an effect which is fairly specific to the soluble MMO system. Inactive soluble extract was fractionated into DEAE-fractions A, B, and C and fraction C compared with fraction C from soluble extract active for the MMO, by using SDS-PAGE (10% gel) (unlike protein A, protein C may not be identified on SDS-PAGE of soluble extracts, and requires DEAE-fractionation in order to be resolved by SDS-PAGE, as does protein B). Protein C appeared much reduced in inactive DEAE-fraction C, as judged by the lack of a band at a molecular weight of 37,000 (see Figure 2.2(b)., and 2.3.A.1.). A similar, but tentative result was also seen with protein B, at a molecular weight of about 16,000. Purification of protein C at these levels of specific activity was not very successful, due to decreased yields, an inability to remove contaminating proteins, and the inherent instability of protein C (see 2.3.C.). For example, despite a 145-fold purification of protein C on a specific activity basis, the final specific activity was 1.6 units (c.f. 6-9 units normally) with major contamination

Figure 2.2. Removal of soluble MMO components by growth on ethene-contaminated methane.



(a) An SDS 5-20% (linear) acrylamide gel of soluble extracts (50 microgram samples of protein) from growth on 0% and 0.06% ethene (v/v with methane), see 2.1.B.2. The culture was switched to ethene contaminated methane(+E) and then back to methane(-E) corresponding with the disappearance and return of the three protein A polypeptides. The gel was prepared by S.J.Pilkington.



(b) SDS-PAGE (10% gel, 50 microgram samples, except track c, 20 micrograms) was performed on fraction Cs prepared from cultures grown in the absence (b,c) and presence (a,d) of ethene. In the presence of ethene, the band at 37,000 molecular weight (protein C) is absent, or much decreased.

from a 43,000 molecular weight protein, and the yield was 13% (c.f. 30-40% normally). Therefore problems experienced with the attempted purification of protein C were at least partly due to its specific absence from soluble extract. For protein C, short term inhibitors did not appear to be present in inactive soluble extracts, since incubation of large amounts of inactive soluble extract with active soluble extract had no effect on protein C activity, as measured by the epoxyethane assay. In this assay of protein C activity, protein C was made rate-limiting by the addition of an excess of proteins A and B, in the form of DEAE-fractions A and B (see Materials and Methods). This result combined with the apparent lack of the MMO components, by SDS-PAGE and attempted protein purification, indicates that the inactivity of soluble extracts is due at least in part to low levels of the soluble MMO components, as opposed to specific inactivation of the MMO components, for example. The concerted disappearance of the three putative MMO components was also more evidence for all three of them being bona fide components of the soluble methane monooxygenase.

In summary, the loss of soluble methane monooxygenase activity from Methylococcus capsulatus (Bath) during the period 21.1.81 to 4.6.81 was due to specific removal of the soluble components from soluble (and crude) extracts. The ability of the cells to grow, albeit more slowly (on ethene-contaminated methane), without apparent A, B, and C proteins, and the insensitivity of whole-cell MMO

activity (assayed with 10 mM formate to provide reducing power, see Materials and Methods) to this inactivation effect might offer support for an alternate MMO system. This might be the particulate MMO, discovered recently by Stanley et al., (1983), but unfortunately this was not detected at this time because sodium thioglycollate was used to stabilize protein C and happens to be a strong inhibitor of the particulate MMO (Stanley et al., 1983).

(2) The cause of inactive soluble extracts

The possibility of defective or contaminated trace elements being the cause of inactive soluble extracts was tested by taking Methylococcus capsulatus (Bath) cultures stored at -80°C, known to have been active for the soluble methane monooxygenase, plating these out and growing them up in shaker flasks with fresh reagents, including trace elements. These cultures were then used as inocula for a 5 litre fermenter, which in turn was used as an inoculum for 100 litre runs, the idea being to produce a large inoculum of cells in exponential phase for the 100 litre fermenter, hopefully with a high specific activity for the soluble MMO.

The trace elements composition was varied, doubling all trace elements except molybdate, and using normal trace elements, and all media included nitrate (0.2%)/mineral salts medium (see Materials and Methods). None of these 100 litre runs gave soluble extract with anything other than low or negligible methane monooxygenase activity, like all of the 100 litre runs carried out during this period. Therefore this problem of inactive soluble extracts did not appear to be due to contamination of the media.

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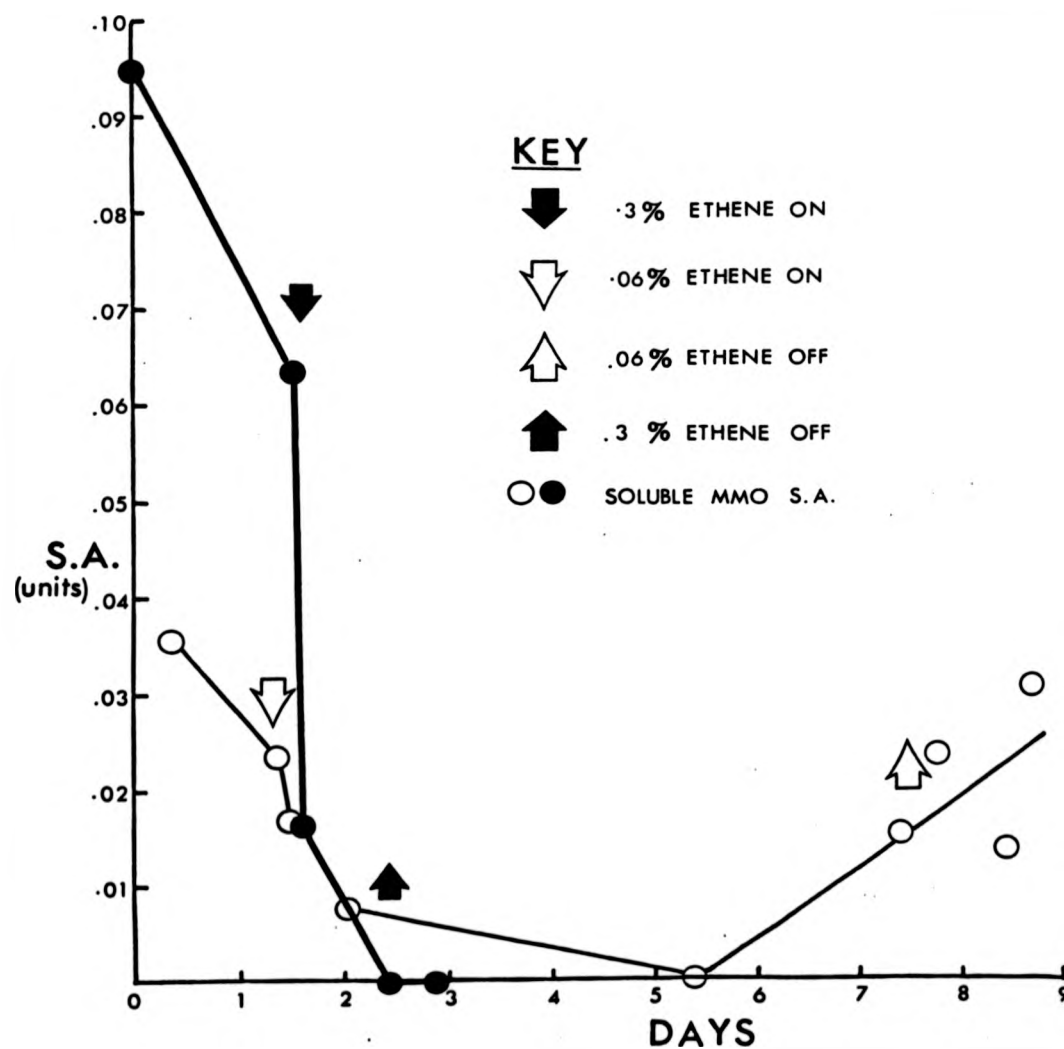
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A contaminant was detected in the technical methane (supplied by the B.O.C.) during an experiment with a katharometer, and was identified as ethene (0.05 to 0.3% v/v in the methane) on the basis of its retention time on Porapak R using a katharometer and on Porapak Q with a flame ionization detector, when the retention times were identical to those of ethene (S.H.Stanley, personal communication). Technical methane is used because it contains 2-4% CO₂, which is a requirement for the initial growth of Methylococcus capsulatus (Bath). Mixing non-contaminated methane from Mogden sewage works with ethene gave a peak profile identical to that seen with B.O.C. methane, and treatment of the B.O.C. methane with gaseous bromine (Colby et al., 1977) removes the contaminating peak seen on Porapak Q using a flame ionization detector, confirming the contaminant as being ethene. The B.O.C. was found to have switched its methane supply from British sewage works to foreign methane at about the same time that soluble extracts inactive for the methane monooxygenase started to be produced. Methylococcus capsulatus (Bath) was then grown on Mogden methane, using a 5 litre fermenter to provide an inoculum for a 100 litre run, and this gave active soluble extract (specific activity 0.06 micromole/min/mg), as have all 100 litre runs since, with ethene-free methane. This evidence indicates that ethene present in the B.O.C. technical methane was responsible for the inability to produce active soluble extract. The observed lack of activity appears to be due to the absence of the soluble

methane monooxygenase components.

Further experiments have been carried out on the removal of methane monooxygenase activity from soluble extracts by ethene-contaminated methane (S.H.Stanley and J.Lund, unpublished work). Methylococcus capsulatus (Bath) was grown up on ethene-free methane and then switched to methane containing 0.06 and 0.3% ethene (v/v in the methane), followed by a return to ethene-free methane (see Figure 2.3.). The degree and rate of removal of soluble methane monooxygenase specific activity appears to be related to the concentration of ethene, though unfortunately in both experiments the specific activity was declining even before the switch to ethene-contaminated methane. With 0.06% ethene the methane monooxygenase activity declined to negligible levels over several days, with cell growth unaffected as monitored by the O.D.₅₄₀ (O.D. = 4.2-4.7 throughout). Activity returned either while the ethene was still present, or about a day after ethene removal, indicating that 0.06% ethene is about the threshold level for this response. SDS-PAGE (5-20%) on soluble extracts indicated that the level of protein A polypeptides usually parallels the methane monooxygenase specific activity (S.H.Stanley, personal communication), so the decline in activity due to cell growth in the presence of ethene is probably due to the absence of the soluble enzyme components in these experiments. With 0.3% ethene the specific activity fell 4-fold in the first hour on contaminated methane, although SDS-PAGE indicated complete protein A

Figure 2.3. The effect on soluble MMO activity of growth on ethene-contaminated methane.

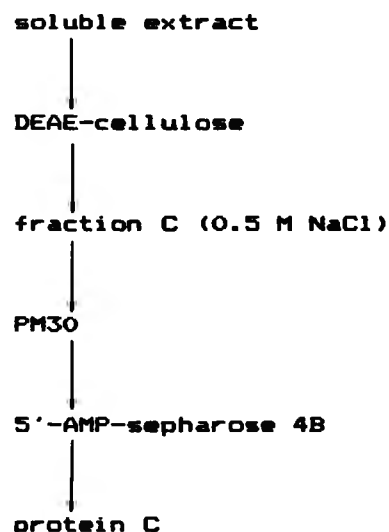


removal only after about 24 hours (S.H. Stanley, personal communication), raising the possibility of removal of methane monooxygenase activity by other means than the amount of enzyme present, e.g. inactivation of the enzyme by epoxyethane or indirect cell-toxicity effects. This level of ethene halted growth of Methylococcus capsulatus (Bath) as indicated by a large and rapid decrease in the O.D.₆₄₀ of the culture. On returning to ethene-free methane the soluble methane monooxygenase activity had not returned after 7 hours (see Figure 2.3.). In summary, there appears to be a general correlation between the presence of ethene and the lack of soluble MMO activity; the reason for the removal of the soluble enzyme components by ethene is not clear, but the ability of Methylococcus capsulatus (Bath) to grow in steady-state growth conditions with negligible detectable soluble components indicates some ethene-induced regulation of the soluble MMO components, through either a direct or indirect effect of ethene.

2.2.A. Purification of protein C of the methane monooxygenase

The methodology used to purify protein C to an acceptable level of homogeneity is that of Colby and Dalton (1978), with minor modifications (see Materials and Methods and Figure 2.4.). These modifications are employed to make the purification as rapid as possible, because protein C has not been found to be stable even with 5 mM sodium thioglycollate as a stabilizing agent (see 2.3.C.) contrary

Figure 2.4. The purification scheme for protein C of the soluble MMO.

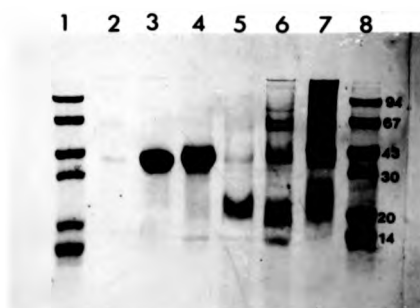


to the result of Colby and Dalton (1978) where protein C was found to be very stable in the presence of sodium thioglycollate. Without thioglycollate, extreme instability of protein C was seen, and therefore thioglycollate was included at all stages of the purification scheme, and not just after the DEAE-cellulose column chromatography step (Colby and Dalton, 1978). Secondly, all steps were performed as fast as possible, e.g. the initial DEAE-fractionation step was accelerated by pumping at a high flow rate, fractionating with small portions of buffers, and the final Sephadex G-100 step was omitted. Fraction C was concentrated by ultrafiltration with a PM30 membrane, because protein C binds the affinity chromatography column

only loosely, so initial fraction C concentration results in higher yields and greater sample purification.

The average mean yield from fourteen fraction C preparations (after the PM30 concentration step) was 40-50%, with a mean purification of 33-fold on a specific activity basis and a purification time from soluble extract of 3.5 to 4.5 hours. This yield is considerably lower than the reported yield of 79% (Colby and Dalton, 1978) or 76% (Colby and Dalton, 1979) but the present purification factor is greater by 1.7 to 2.2-fold. Since the specific activity of the present soluble extract is about half that reported previously (Colby and Dalton, 1979), the resulting DEAE-fraction C preparations have a similar specific activity. The yield from the affinity chromatography step was 76%, with around 2.5 to 3.5-fold purification as indicated by the increase in specific activity, and is similar to the reported yield of 59% and 3.2-fold purification for this 5'-AMP-sepharose 4B step (Colby and Dalton, 1979). SDS-PAGE of the relevant fractions indicated a high degree of homogeneity for protein C (see Figure 2.5.), greater than or equal to 95%, in agreement with Colby and Dalton (1979). At this stage protein C decays at between 1% and 5% per hour, and so was not subject to Sephadex G-100 column chromatography, which was used by Colby and Dalton (1979) to remove the final light contamination. Overall the purification steps for protein C take around 6 to 6.5 hours, so the overall purification time from harvesting of the cells to production of pure protein C

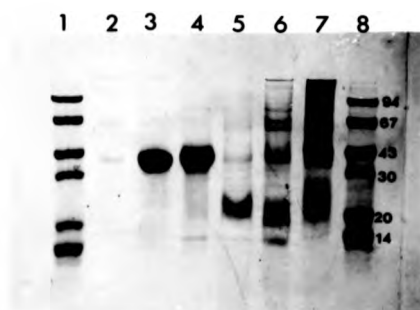
Figure 2.5. The purity of protein C by SDS-PAGE.



SDS-PAGE (10% gel) was carried out on fractions eluting from the 5'-AMP-sepharose 4B column. Tracks 1 and 8 are low molecular weight markers (50 micrograms protein), track 7 is DEAE-fraction C (50 micrograms), and tracks 2 to 6 are column eluates (up to 50 micrograms), in some cases lightly loaded, due to the dilute nature of the sample. The fractions giving tracks 3 and 4 were used as pure protein C. Tracks 2 to 6 are numbered opposite to the order in which they elute from the affinity chromatography column.

is between 10.5 to 11 hours. A circa 100-fold purification is achieved with 30 to 40% yield, which is very similar to the reported final yield of 35% (Colby and Dalton, 1979, see Table 2.2.). The protein C, at between 4 mg/ml and 8 mg/ml was found to be concentrated enough for all the work undertaken in this project.

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Table 2.2. Previous and present purification data on protein C.

| stage | soluble extract | fraction C* | AMP-sepharose eluate |
|----------------|-----------------|-------------|----------------------|
| volume | 250 | 15** | 11 |
| (ml) | (240) | (128) | (16.6) |
| protein | 20,000 | 271 | 68 |
| (mg) | (17,700) | (900) | (165) |
| activity | 1,200 | 540 | 408 |
| (units) | (2130) | (1620) | (960) |
| specific | .06 | 2.0 | 6.0 |
| activity | (.12) | (1.8) | (5.8) |
| (units/mg) | | | |
| yield | 100 | 45 | 34 |
| (%) | (100) | (76) | (45)*** |
| purification 1 | | 33 | 99 |
| factor | (1) | (15) | (48) |

The bracketed numbers are those of Colby and Dalton (1979), for comparison.

* The fraction C data is given after the PM30 concentration step, for recent preparations.

** In practice, two fraction Cs are pooled at this stage (see Materials and Methods), but data is given for only one fraction C preparation, for the sake of easier comparison with the earlier data.

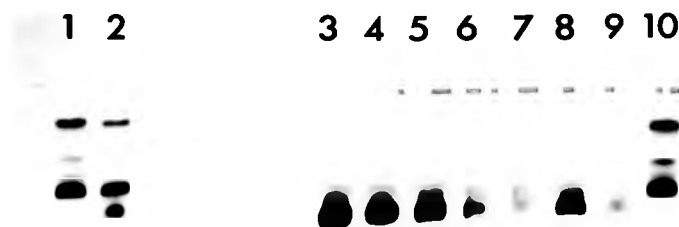
*** The final reported yield after the Sephadex G-100 step.

2.2.8. Crit

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Figure 2.6. Non-denaturing acrylamide gel electrophoresis of protein C.



Protein C was electrophoresed on a 5% non-denaturing acrylamide gel with low molecular weight markers. Tracks 1 and 10 were with standards (50 micrograms protein), and track 2 was with standards (30 micrograms) and protein C (20 micrograms). Tracks 3, 4, 5, 6, and 7 were a protein C at loadings of 50, 40, 30, 20, and 10 micrograms respectively, and tracks 8 and 9 were another protein C preparation at 30 and 10 micrograms of protein respectively.

Figure 2.6. Non-denaturing acrylamide gel electrophoresis of protein C.



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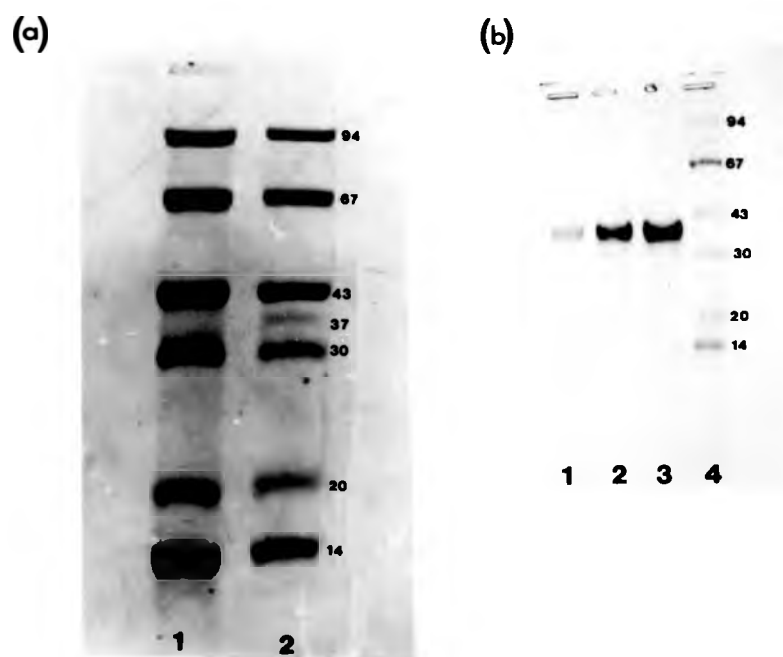
activities. In addition, recent protein C preparations share many other properties with preparations previously reported, very similar optical and EPR spectra, a similar molecular weight by denaturing gel electrophoresis, for example, and therefore appears to be the protein C of Colby and Dalton (1978, 1979). Further evidence corroborating this similarity comes from using antibodies prepared to a fairly pure sample of protein C produced at that time; rabbit IgG anti-protein C antibodies were linked covalently to Sepharose 4B to give an affinity chromatography column which bound protein C from fraction C (see Materials and Methods), as indicated by the presence of a yellow band which eluted with urea and had the same molecular weight by SDS-PAGE as recent protein C preparations (37,000), so shares antigenic determinants with the previous protein C.

2.3. A basic characterization of protein C

2.3.A.1. Polypeptide molecular weight

The polypeptide molecular weight was estimated by SDS-PAGE on 5%, 7.5%, and 10% acrylamide gels using low molecular weight range markers at pH 7, and gave a value of $37,000 \pm 2,000$, using the LKB flatbed system (Figure 2.7., see Materials and Methods). This value is in good agreement with the molecular weight estimate of Colby and Dalton at 39,000 using 10-30% acrylamide exponential-gradient slab gels with 0.1% SDS at pH 8.5.

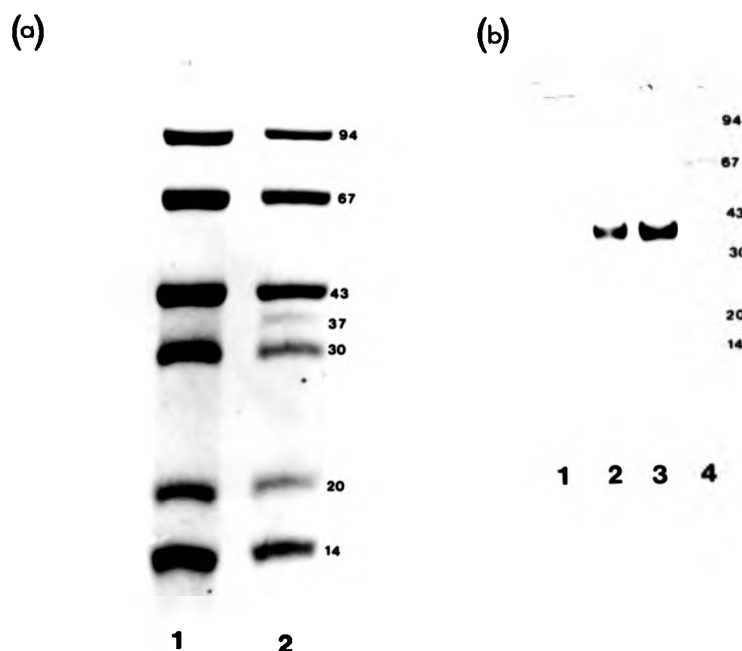
Figure 2.7(a,b). The polypeptide molecular weight of protein C by SDS-PAGE.



(a) SDS-PAGE was carried out with a 5% acrylamide gel. Track 1 was low molecular weight markers (50 micrograms protein), track 2 was the same amount of marker proteins together with protein C (10 micrograms).

(b) SDS-PAGE was carried out with a 7.5% acrylamide gel. Track 4 was low molecular weight markers (30 micrograms protein), and tracks 1, 2, and 3 were 8, 20, and 40 micrograms of protein C respectively.

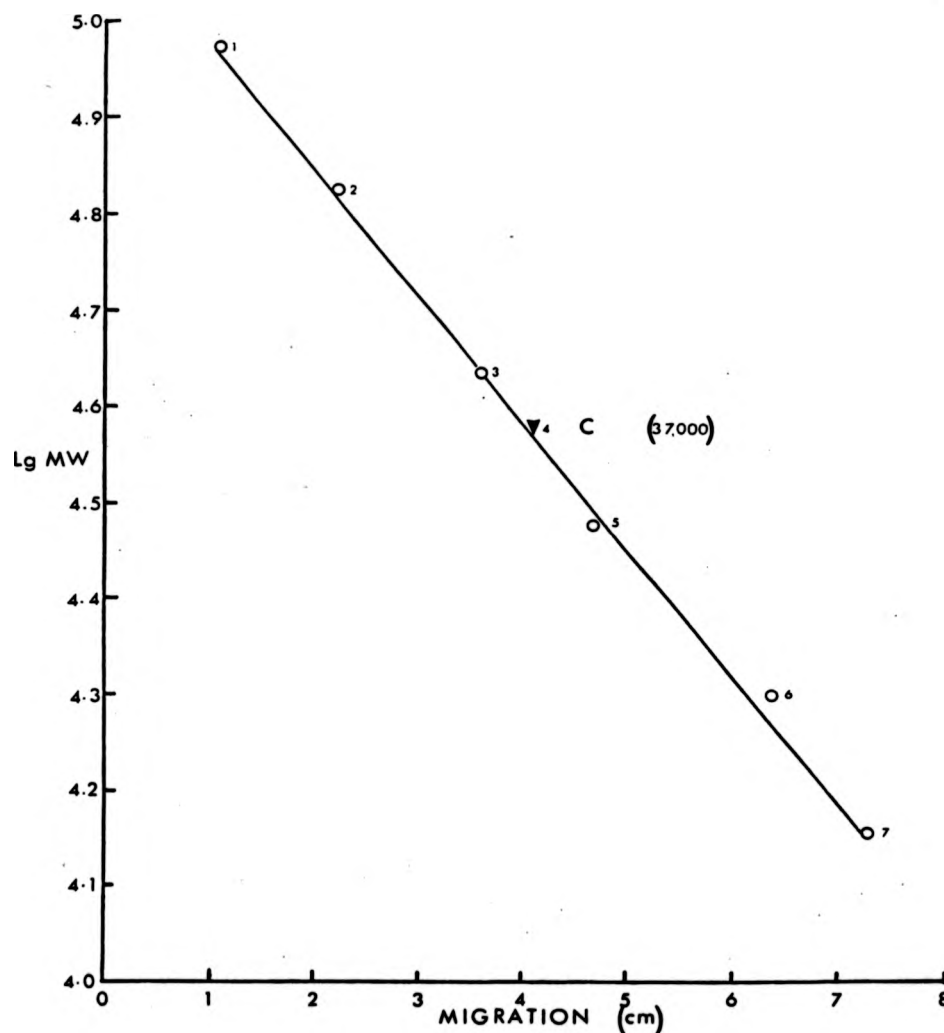
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(b) SDS-PAGE was carried out with a 7.5% acrylamide gel. Track 4 was low molecular weight markers (30 micrograms protein), and tracks 1, 2, and 3 were 8, 20, and 40 micrograms of protein C respectively.

Figure 2.7(c). The molecular weight of protein C by SDS-PAGE.

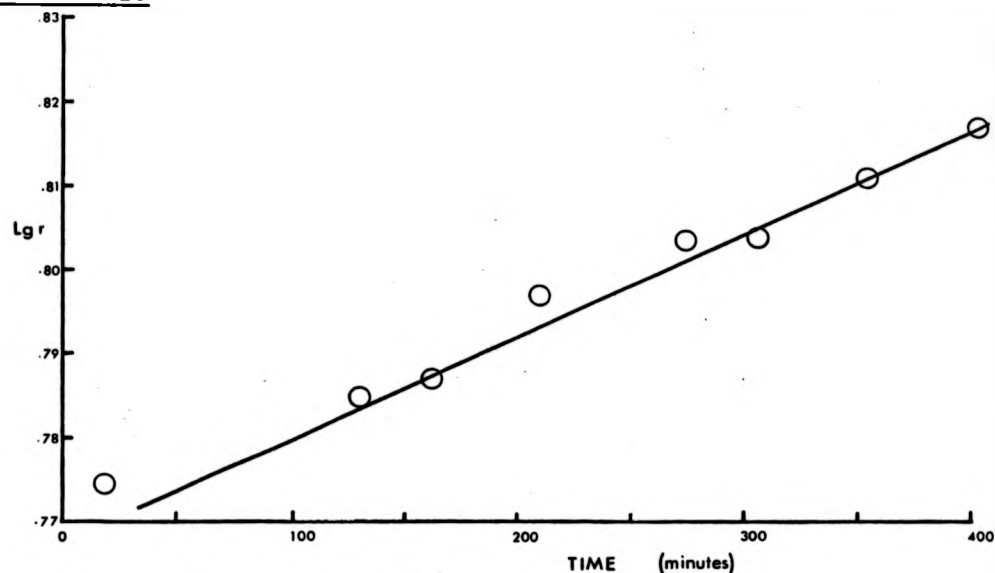


SDS-PAGE was performed on protein C (4) and low molecular weight markers (Figure 2.7(a)). The molecular weight standards were (1) phosphorylase b (94,000); (2) B.S.A. (67,000); (3) ovalbumin (43,000); (5) carbonic anhydrase (30,000); (6) soybean trypsin inhibitor (20,100); (7) alpha-lactalbumin (14,400).

2.3.A.2 The associative properties of protein C

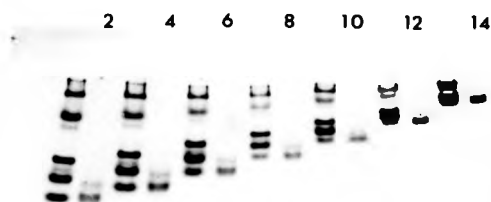
Colby and Dalton (1978) reported that the molecular weight of protein C by gel-filtration against standards on Sephadex G-100 was 44,600, indicating that protein C was a monomer. The putative monomeric nature of protein C was checked by sedimentation velocity ultracentrifugation, using a Beckman model E analytical ultracentrifuge, and yielded an $S_{20,w}$ value of 2.96×10^{-13} second (see Figure 2.8., and Materials and Methods), which is empirically consistent with a molecular weight of around 32,000, assuming that the partial specific volume is not abnormal (Atassi and Gandi, 1965). Therefore this data is consistent with protein C being a monomer, as proposed by Colby and Dalton (1978). Further evidence that protein C is a monomer comes from non-denaturing electrophoresis using the method of Lambert and Fine (1979), where the rate of migration of protein C relative to molecular weight standards indicated a molecular weight of $\leq 30,000$, (Figure 2.9., also see 2.3.C.). A 74,000 molecular weight dimer would have a very different mobility from those observed, indicating that protein C is monomeric by this technique. The presence of multiple bands under non-stabilizing conditions indicates protein C forms with different electrophoretic properties, in contrast to the results seen under conditions of functional stabilization. In summary, three separate techniques indicate that protein C is a monomer.

Figure 2.8. Sedimentation plot to estimate the S value for protein C.



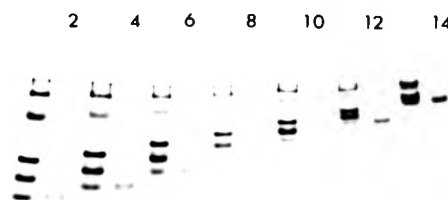
The slope of this plot ($1.246 \times 10^{-4} \text{ minute}^{-1}$, estimated by the method of least squares), yields the S_{obs} , the observed sedimentation coefficient, since $S_{obs} = dLnr/dt / w^2$. The $S_{20,w}$ was then estimated by use of a viscosity coefficient correction, $S_{20,w} = S_{obs} \times \eta_4/\eta_{20,w}$.

Figure 2.9. Molecular weight of protein C by non-denaturing electrophoresis.



A vertical gel electrophoresis system with a 5% to 30% linear acrylamide gel was used, as detailed in Materials and Methods. Samples (20 micrograms of protein) were loaded at times 0, 1, 2, 3, 4, 5 and 6 hours, corresponding to tracks 2, 4, 6, 8, 10, 12 and 14 respectively, and then left for two hours. Each sample was loaded together with adjacent low molecular weight markers (50 micrograms protein). The rate of migration of protein C may be interpreted to yield an estimate of the molecular weight, using the fact that the square root of the running time is directly proportional to the migration distance.

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2.3.A.3. The isoelectric point of protein C

Isoelectric focusing was carried out with protein C on acrylamide gels (Materials and Methods), staining with Coomassie Blue and also for NADH-acceptor reductase activity using Nitro Blue Tetrazolium and NADH (on non-denaturing gels, Colby and Dalton, 1979), which gives rise to a purple colouration when positive, and is otherwise colourless. Denaturing isoelectric focusing using pH range 3-10 ampholytes gave one major intense band with a $pI = 4.8 \pm 0.2$ and two close minor bands (Figure 2.10(a).), these latter having about 10% of the total intensity by gel-densitometry. With Pharmalyte ampholytes of pH range 4-6.5 on non-denaturing gels, one sharp band was found on staining with Coomassie Blue (Figure 2.10(b).), indicating protein C purity at least over this pH range. The position of the band indicated a $pI = 4.8 \pm 0.2$, and on one gel up to 5 closely spaced bands were seen. With NADH and Nitro Blue Tetrazolium one single intense purple band was seen (Figure 2.10(b).), corresponding with the protein stained band, indicating that protein C had retained its acceptor-reductase activity. However on one lightly loaded gel the single band was resolved into several fine bands, probably due to variant forms of protein C, (or possibly other proteins). Similarly, isoelectric focusing over the range 3.5-8.5 gave 5 bands in the range 4.6-5.2 with three major bands and the other two (most acidic) minor bands gave no Nitro Blue Tetrazolium stain, so appeared to lack NADH-acceptor reductase activities, perhaps because the FAD

Figure 2.10. Isoelectric focusing of protein C on cylinder gels.

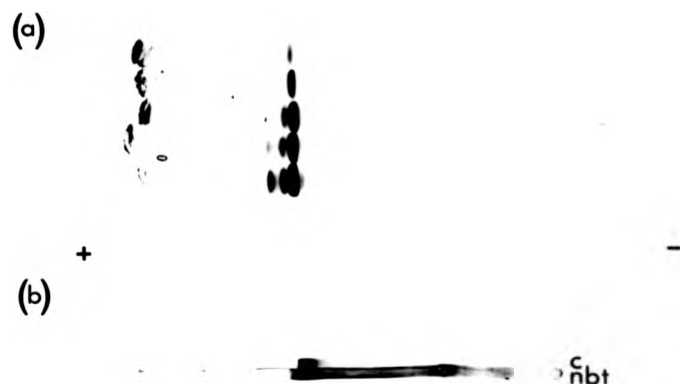


(a) Protein C (10, 20, 30, 50, and 100 micrograms) was focused under denaturing conditions, with 8 M urea and 3% Nonidet P-40, using 3.2% ampholines and a 6% acrylamide gel.

The gels were focused for 5,600 volt hours.

(b) Protein C (50 micrograms) was focused under non-denaturing conditions with 2.3% pharmalytes on 3.2% acrylamide gels, for 2000 volt hours. Gels were stained for N.B.T. activity (nbt) and Coomassie stained (c).

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The gels were focused for 5,600 volt hours.

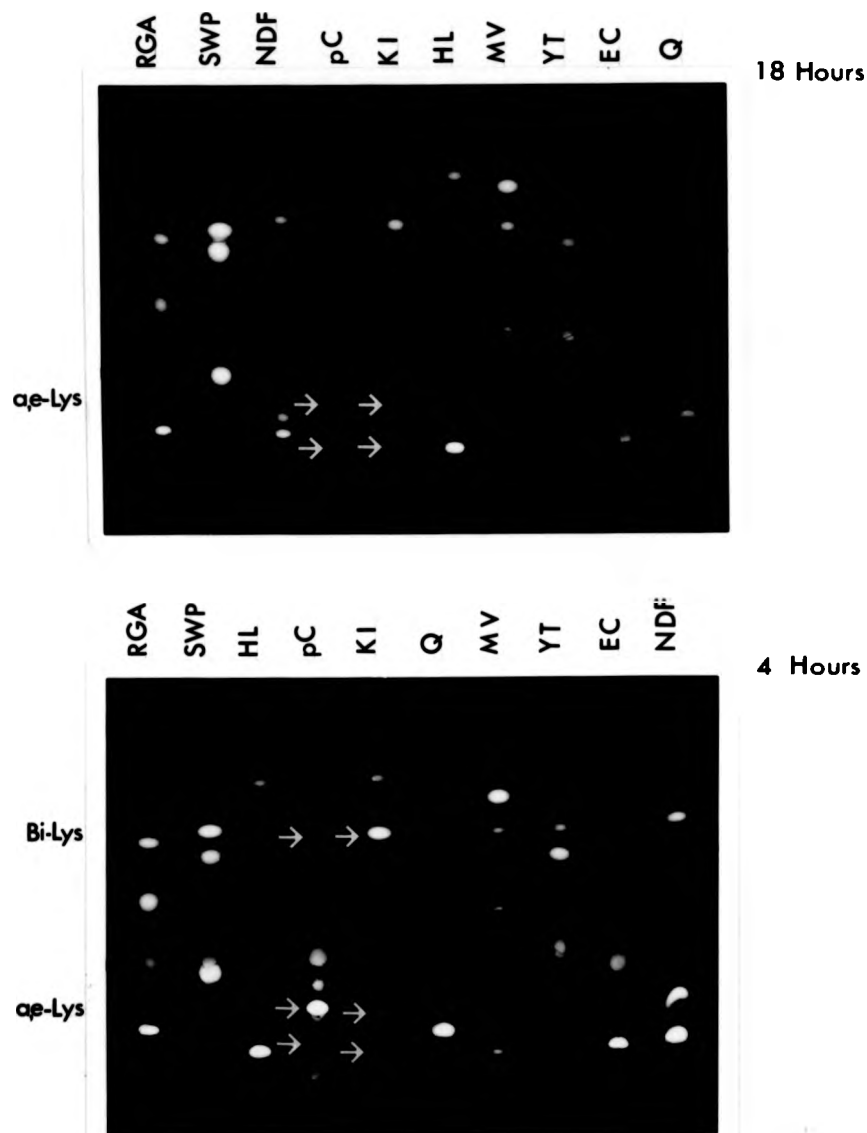
(b) Protein C (50 micrograms) was focused under non-denaturing conditions with 2.3% pharmalytes on 3.2% acrylamide gels, for 2000 volt hours. Gels were stained for N.B.T. activity (nbt) and Coomassie stained (c).

group was absent (see 3.2.); also, during the running of the samples both yellow-brown and orange-red bands were seen, the orange-red band might be protein C lacking an FAD centre, but with an iron-sulphur centre, since iron-sulphur centres of this type are usually reddish in colour (see Malkin, 1973). It appears that protein C has variant forms as indicated by non-denaturing isoelectric focusing, probably due to the loss of prosthetic groups and the resultant charge alterations on protein C. This loss would appear to be consistent with the instability of protein C and the stoichiometries for occupancy of protein C by its prosthetic groups (see 2.3.C.).

2.3.A.4. N-terminal analysis of protein C

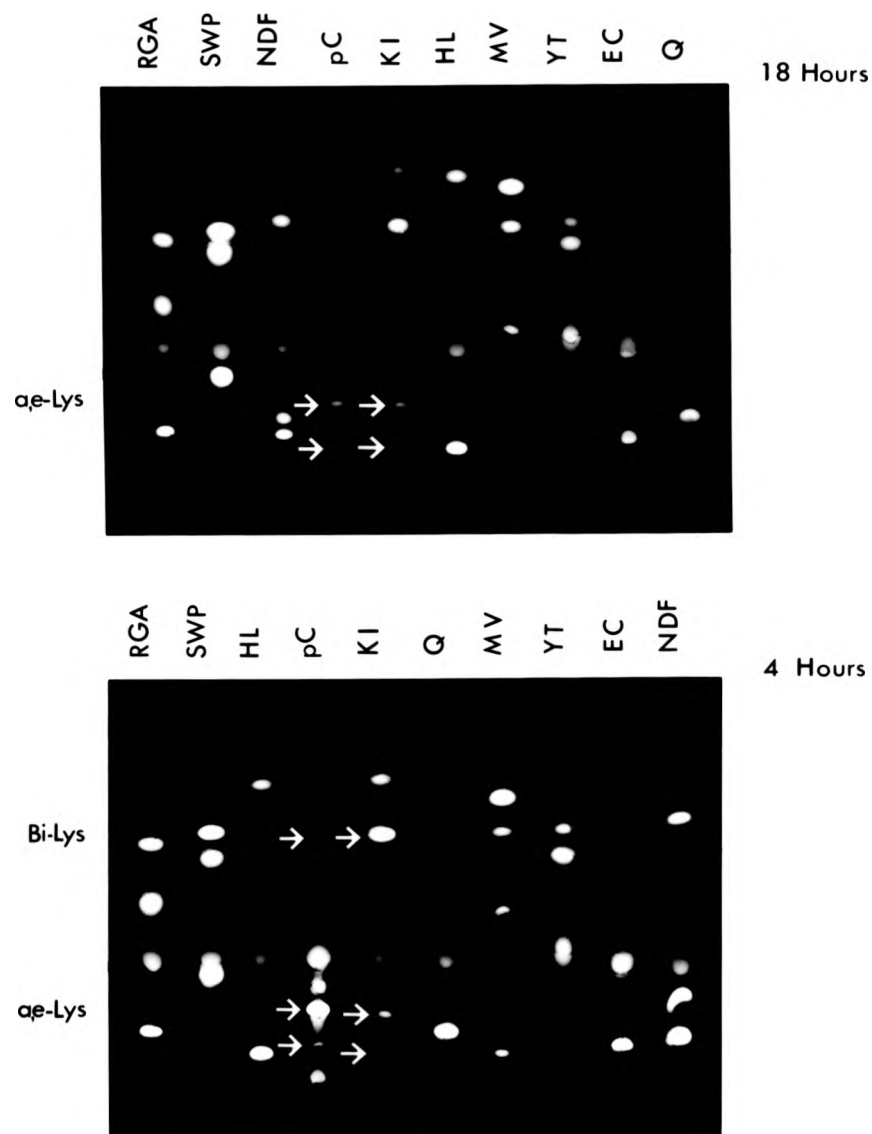
Protein C (0.5 mg) was dansylated with an excess of dansyl chloride in the presence of deionized urea to unfold the protein, and sodium bicarbonate to raise the pH. This treatment places the fluorescent dansyl moiety on the N-terminal amino acid residues of proteins present in the sample. Acid hydrolysis for 4 and 18 hours was then used to liberate this derivative, which was identified by 1-dimensional thin-layer chromatography against standards, with 2 solvent systems (Figure 2.11., see Materials and Methods). After the first solvent system (benzene:pyridine:glacial acetic acid) the sample fluorescence had not moved from the origin, indicating the presence of a polar N-terminal amino acid residue. After the second solvent system (n-butanol saturated with 0.2 M

Figure 2.11. The N-terminal analysis of protein C by Dansylation.



NaOH), the presence of spots corresponding to alpha Lys-dans, eta Lys-dans, and Lys bis-dans (after 4 hours hydrolysis only, because this product is unstable to acid hydrolysis) and an unknown Lys-dans product indicated that the N-terminal amino acid residue of protein C was probably lysine.

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Key to Figure 2.11.

| | |
|----|-----------------------|
| R | Arginine |
| G | Glycine |
| A | Alanine |
| S | Serine |
| W | Tryptophan |
| P | Proline |
| N | Asparagine |
| D | Aspartate |
| F | Phenylalanine |
| pC | Protein C hydrolysate |
| K | Lysine |
| I | Isoleucine |
| H | Histidine |
| L | Leucine |
| M | Methionine |
| V | Valine |
| Y | Tyrosine |
| T | Threonine |
| E | Glutamate |
| C | Cysteine |
| Q | Glutamine |

The single letter abbreviations represent dansyl derivatives of the respective amino acids.

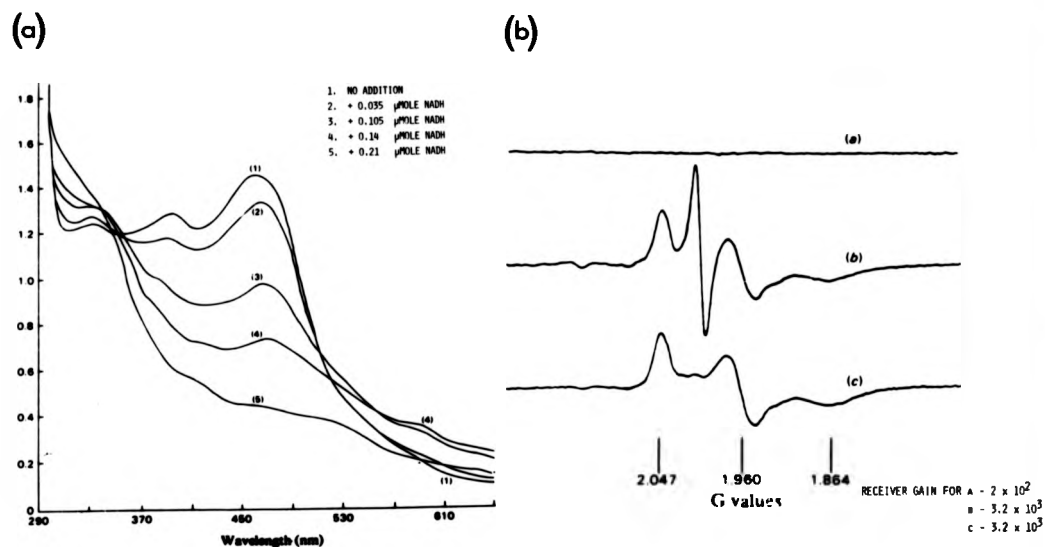
2.3.B. The prosthetic groups of protein C

(1) The nature of the prosthetic groups of protein C

(a) Protein C was reported to contain two redox centres, a flavin group (FAD) (Colby and Dalton, 1978), and an iron-sulphur centre (Fe_2S_2) (Colby and Dalton, 1979). Both these centres were assumed to be prosthetic groups, i.e. firmly bound coenzymes which are directly involved in enzymic catalysis.

Colby and Dalton (1978) found that a fluorescent yellow substance could be liberated from protein C by boiling and centrifugation to remove denatured protein. The electronic absorption spectrum of the substance was like that of FAD, with maxima at 260 nm, 375 nm, and 445 nm. The fluorescence excitation and emission spectra were also identical to that of authentic FAD, with excitation maxima at 370 nm and 450 nm and a single emission maximum at 518 nm. Thin-layer chromatography on silica gel with two different solvent systems showed the substance from protein C to have identical R_f values to those of authentic FAD (Colby and Dalton, 1978). Therefore the substance was thought to be the flavin FAD, and this was proposed as a prosthetic group for protein C. On partial reduction with NADH under anaerobic conditions, semi-reduced protein C displayed a new spectral species in the 570-630 nm region, which disappeared on further reduction with NADH, and the spectral band was also seen in the absence of NADH on partial photoreduction (with 20 mM EDTA) of protein C under anaerobic conditions (see Figure 2.12(a)). This behaviour was inferred to be due to the formation of a neutral flavin

Figure 2.12. Redox titrations of protein C by spectrophotometry and E.P.R.



(a) Protein C (5.5 mg/ml) was reduced with NADH under anaerobic conditions (Colby and Dalton, 1978), with reduction monitored in a spectrophotometer.

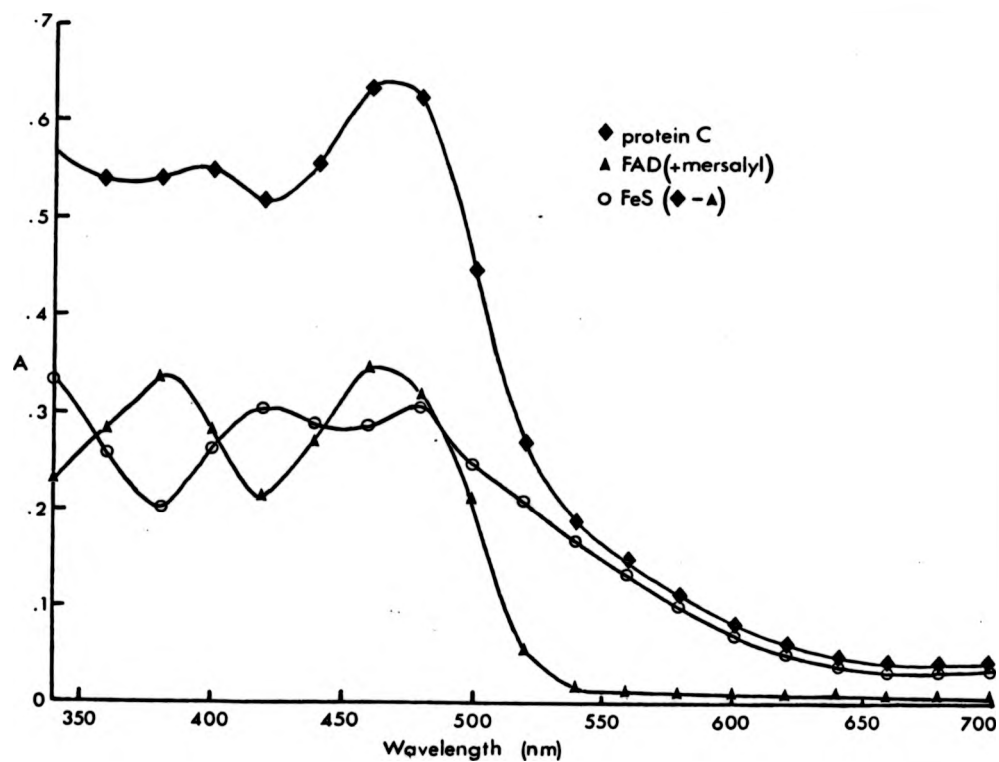
(b) E.P.R. spectra of the aerobic reduction of oxidised protein C (a), with excess NADH (b), and then dithionite (c) (Colby and Dalton, 1979). The conditions were 19 Kelvin and 10 mW (a) and 1 mW (b,c) microwave power.

semiquinone (blue) (Müller et al., 1972). An anionic semiquinone (red) would not be expected to have appreciable absorbance at wavelengths greater than around 600 nm. Further support for formation of a semiquinone came from EPR studies, since reduction of protein C gave rise to a free-radical signal at $g = 2.002$, which disappeared on further reduction with sodium dithionite, and was thought to be due to the semiquinone form of the FAD redox centre (Colby and Dalton, 1979, see Figure 2.12(b)).

(b) Protein C was reported to contain iron, between 1.3 and 1.5 atoms per polypeptide (based on a molecular weight of 42,000). The iron was liberated by boiling with 1% (w/v) SDS for 10 minutes and assayed with bathophenanthroline, and was thought to be tightly bound because in the absence of SDS no colour development occurred in the iron assay (Colby and Dalton, 1978). Later, protein C preparations were obtained by an improved purification procedure that had 1.92 iron atoms per polypeptide (using a molecular weight estimate for protein C of 44,600). In addition 2.06 moles of "acid-labile" sulphide were detected per mole of protein C (so named because under acidic conditions H_2S is liberated from iron-sulphur centres), indicating the presence of an iron-sulphur centre. Reduction of protein C with NADH gave rise to an E.P.R. signal of the plant ferredoxin type, with rhombic symmetry and g -values at 2.047, 1.960, and 1.864. Further reduction with sodium dithionite did not alter the signal (see Figure

2.12(b)). These data were consistent with the presence of an Fe_2S_2 centre, and were consistent with the stoichiometric data for iron and "acid-labile" sulphide. Core extrusions (a method in which a thiol agent is used to displace the iron-sulphur centre from proteins into an aprotic solvent) were taken to indicate the presence of an Fe_2S_2 centre as opposed to an Fe_4S_4 centre, which would possess very different spectral characteristics, and indicated that protein C had 0.96 iron-sulphur centres per polypeptide chain (Colby and Dalton, 1979). These authors had therefore satisfied several of the criteria required for identification of the iron centre as an Fe_2S_2 centre, namely no EPR signal in the oxidized state, a broad and rhombic spectrum of the plant ferredoxin type which underwent no further qualitative changes with reduction, with a g-value of greater than 2 in the reduced state, and characteristic extrusion products. Another criterion is a characteristic optical spectrum (see Xavier *et al.*, 1981), which can be seen for protein C on treatment with the mercurial thiol mersalyl, used generally to destroy iron-sulphur centres (Malkin and Rabinowitz, 1966), as have other mercurials (Lovenberg *et al.*, 1963). Mersalyl bleaches protein C from yellow-brown to yellow, and the difference spectra of native and mersalyl treated protein C indicate a broad and significant absorbance due to the iron-sulphur centre (see Figure 2.13.), approximately $46\% \pm 7\%$ of the total absorbance at 465 nm, for example. The resulting iron-sulphur spectrum is very similar to other iron-sulphur

Figure 2.13. Optical contributions of the redox centres of protein C.



Protein C (50 nmoles) in 0.69 ml of 20 mM Tris-HCl pH 7, was treated with 0.0125 ml of mersalyl (860 nmoles) in 0.5 M Tris-HCl pH 7.6.

proteins of the Fe_2S_2 type, with a broad wavelength absorbance with maxima at 340 nm, 420 nm, and 480 nm, and similar ratios for the extinction coefficients at these wavelength, see 3.1.A. The spectrum does not resemble that of FAD, and no spectral change is seen on addition of a large excess of mersalyl to solutions of FAD. In summary, there appears to be good evidence that protein C has two types of redox centre; an FAD and an Fe_2S_2 centre.

(2) The prosthetic group stoichiometries of protein C

(a) Estimates of protein C concentration

The estimated stoichiometries for the prosthetic groups of protein C appear to be dependent on the method used to estimate the stoichiometry and on the estimate of the molar protein concentration. Protein concentration is in turn a function of the estimate of the molecular weight of the protein and its concentration on a weight basis. Therefore present and previous estimates of protein C concentration will be discussed before considering protein C prosthetic group stoichiometries. Any estimations of the redox centres:protein stoichiometries are obviously critically dependent on an accurate estimation of the true protein concentration, but with protein C different protein assays (see Materials and Methods) appear to give different results for the protein C concentration (on a mg/ml basis) referencing against the O.D.₄₄₀ of protein C, see Table 2.3.

Table 2.3. [Protein C] estimates by different assays.

| method | [Protein C] giving O.D. ₄₄₀ = 1 |
|---------------|--|
| Lowry | 3.8* mg/ml |
| Bio-Rad | 2.1 mg/ml |
| Precipitation | 4.2 mg/ml |

* Both previous and present estimates by this method are the same.

Colby and Dalton (1978, 1979) used the method of Lowry, with dried crystalline bovine plasma albumin as a standard. Present estimates of protein C using the method of Lowry agree with previous work (Colby and Dalton, 1978, 1979), since the O.D.₄₄₀ for a given protein concentration is the same, about 3.8 mg/ml giving an O.D.₄₄₀ of 1. Since both prosthetic groups make a significant optical contribution at this wavelength (see 2.3.B.1.), these data also indicate that the overall prosthetic group:polypeptide stoichiometry is likely to be very similar for both previous and present protein C preparations. The method of protein precipitation appears to give a slightly larger value for the amount of protein per ml giving an O.D.₄₄₀ of 1, and this value is almost exactly double that found with the Bio-Rad protein assay. The value found using the precipitation method has been used for estimates of the molar concentration of protein C in this work, because it is an absolute method for

the determination of the amount of protein and not relative to a standard such as bovine serum albumin. Therefore there is a considerable variation in the estimate of protein C concentration depending on the method used. Colby and Dalton (1978, 1979) calculated the molar protein C concentration using molecular weight estimates of 42,000 and 44,600 respectively. This last value was the molecular weight determined on a size basis using Sephadex G-100, as opposed to the SDS-PAGE estimate of 39,000 by these authors.

The present author feels that a molecular weight estimate based on SDS-PAGE is more accurate than a size based estimate, so uses a molecular weight estimate of 37,000 (see 2.3.A.1.). This gives a larger estimate of the molar protein C concentration for a given weight/ml of protein than the previous authors obtained, resulting in correspondingly decreased prosthetic group:protein C stoichiometries (see Table 2.4.).

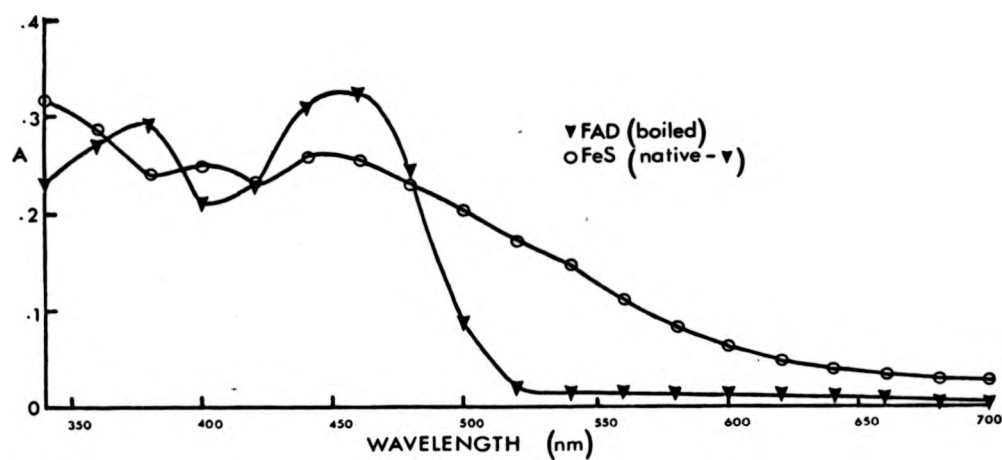
(b) The FAD:protein C stoichiometry

Initially, the FAD concentration was estimated in two ways (Colby and Dalton, 1978), by boiling protein C and estimating the concentration of FAD (which is heat stable) at 445 nm* and by using the absorbance of protein C at 465 nm, giving stoichiometries of 0.85:1 and 1:1 for the FAD:protein C ratio (protein was estimated by the method of Lowry with a molecular weight estimate of 42,000). However, use of the protein C absorbance at 465 nm will give an overestimate of the amount of FAD present because the

* $E_{445} = 11.3 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$; see Colby and Dalton, 1978.

iron-sulphur centre makes a very substantial contribution at 465 nm (typically 46% of the total peak height). Also, boiling protein C in the absence of SDS only partially removes this contribution (see Figure 2, Colby and Dalton, 1978, where the 465 nm contribution after boiling is 77% of the initial total O.D.₄₆₅). This result is different from that seen by boiling protein C in 1% SDS (Colby and Dalton, 1979, also see Figure 2.14., which indicates that boiling in SDS totally removes the absorbance due to the iron-sulphur centre and is similar in effect to treatment with mersalyl (mersalyl addition gives no further absorbance decrease). Therefore any estimate of the amount of FAD present will be an overestimate. The resultant alternative values to these stoichiometries are given in Table 2.4., using the protein C concentration estimates made then (Colby and Dalton, 1978, 1979) and presently. The alternative values come out at around 0.54 to 0.60, using previous estimates for the protein C concentration. Figure 1 (Colby and Dalton, 1979) indicates that if all the absorbance at this maximum was due to FAD the stoichiometry would be 0.94:1, but a very substantial portion of the absorbance in both Figures of part (b) is due to the iron-sulphur centre, as can be seen either by treatment with mersalyl, or boiling with 1% SDS to destroy this centre, see Figure 2.14. Both Figures 1 and 2 (Colby and Dalton, 1979) are consistent with a protein C concentration of 3.8 mg/ml (by the method of Lowry) for an O.D.₄₆₅ of 1 and using the quoted molecular weight estimate for protein C of 44,600 the alternative stoichiometry is 0.62, in agreement with the previous alternative

Figure 2.14. Destruction of the iron-sulphur centre by boiling with 1% SDS.



Protein C (67 nmoles in 1 ml of 50 mM sodium phosphate buffer, pH7) was boiled with 1% SDS for 10 minutes in the dark (Colby and Dalton, 1979).

stoichiometry of 0.54 to 0.60. When these figures are altered to present values for the protein C concentration, based on a molecular weight of 37,000 and 4.2 mg/ml giving an $O.D._{445} = 1$, the FAD:protein C ratio is around 0.42, in agreement with present findings on protein C (obtained by boiling with 1% SDS to destroy the iron-sulphur centre and to release the FAD, Figure 2.14., and by treatment with mersalyl to destroy the iron-sulphur centre, see Figure 2.13.). This stoichiometry is consistent with previous and present protein C preparations being quantitatively very similar with respect to the amount of FAD present. However, the quoted stoichiometry is 1:1 by boiling with 1% (w/v) SDS (Colby and Dalton, 1979), but for this to hold 0.043 mM protein C would have to have nearly double the $O.D._{445}$ of 0.043 mM FAD (see Figures 1 and 2, Colby and Dalton, 1979) due to the iron-sulphur centre absorbance, and this is clearly not the case, indicating that a 1:1 stoichiometry does not hold. It might be argued that FAD is altered with respect to absorbance on binding protein C, but this does not seem to be the case because mersalyl treated protein C (in aqueous solution and in hexamethyl phosphoramide) and protein C boiled with 1% (w/v) SDS have very similar maximum absorbances in this region, indicating against any large change in maximum absorbance on liganding of FAD to protein C. These data are summarized in Table 2.4.

(c) The iron-sulphur:protein C stoichiometry.

The iron-sulphur centre may be estimated from the

optical contribution at 465 nm (obtained by boiling with SDS or by treatment with mersalyl to destroy this centre, see Figures 2.14. and 2.13.). For example, using likely values for a mM extinction coefficient of 9.9₄₆₅ (Xavier *et al.*, 1981), and subtracting the absorbance due to the FAD, the stoichiometry is about 0.42 iron-sulphur centres per protein C polypeptide with present estimates of the protein concentration and 0.56 with previous estimates. Core extrusion data indicated 0.96 iron-sulphur centres per protein C using previous estimates of protein C concentration (Colby and Dalton, 1979), a considerable discrepancy from the 0.56 quoted above, but indicating that a likely range for this stoichiometry is 0.42:1 to 0.96:1. However, core extrusion experiments also give absorbances of the same order with extraneous iron (and sulphide), and have been proposed as a rapid assay for total (extrudable) iron (Gillum *et al.*, 1977). The presence of iron (and sulphide) extraneous to intact iron-sulphur centres might offer an explanation of the above discrepancy, and would also explain why iron (and sulphide) were found in similar amounts to the extruded core. The iron and sulphide found (Colby and Dalton, 1979) are also given in Table 2.4., together with alterations due to the different protein C concentration estimate used presently (recently, one mole of iron per mole of protein C has been found, in reasonable agreement with other present estimates of the iron-sulphur centre stoichiometry, but only half of the previous estimate). Inspection of this Table indicates that likely ranges for

Table 2.4. Estimates of protein C prosthetic group stoichiometries.

| | Colby and Dalton 1978 | Colby and Dalton 1979** | this work* |
|---|-----------------------|-------------------------|------------|
| FAD:protein | 0.85:1 1:1 | 1:1 | 0.42:1 |
| Fe ₂ S ₂ :protein | | 0.96:1 | 0.41:1 |
| Iron:protein | 1.3-1.5:1 | 1.92 | 1:1 |
| sulphide:protein | | 2.06:1 | |

The protein C preparations used for these stoichiometric estimates had specific activities of around 6 micromoles ethylene oxide minute⁻¹ mg protein⁻¹.

Table 2.4. Estimates of protein C prosthetic group stoichiometries.

| prosthetic group | stoichiometry | method | ref. |
|---|-----------------------|----------------------|------|
| FAD:protein | 0.85:1 | boiling | 1 |
| | <u>(0.67:1)*</u> | | |
| | 1:1 | O.D. 445 | 1 |
| | <u>(0.79:1)*</u> | | |
| | 1:1 | boiling (SDS) | 2 |
| | <u>(0.75:1)*</u> | | |
| | 0.42:1 | mersalyl (465 nm) | 3 |
| | <u>[0.56:1]**</u> | | |
| | 0.42:1 | boiling (SDS) | 3 |
| | <u>[0.56:1]**</u> | | |
| Fe ₂ S ₂ :protein | 0.96:1 | core extrusion | 2 |
| | <u>(0.72:1)*</u> | | |
| | 0.41:1 | mersalyl (465 nm) | 3 |
| | <u>[0.55]**</u> | | |
| iron:protein | 1.3-1.5:1 | bathophenanthroline | 1 |
| | <u>(1.04-1.19:1)*</u> | | |
| | 1.92 | bathophenanthroline | 2 |
| | <u>(1.44)*</u> | | |
| | 1.0:1 | bathophenanthroline | 3 |
| | <u>[1.34:1]**</u> | | |
| sulphide:protein | 2.06 | acid-labile sulphide | 2 |
| | <u>(1.46)*</u> | | |

Key to Table 2.4.

* Indicates alternative estimates of stoichiometries based on a molecular weight of 37,000 and a protein C solution of 4.2 mg/ml (precipitation method) giving an O.D.₄₄₀ of 1.

** Indicates alternative estimates of stoichiometry based on a molecular weight of 44,600 and a protein C solution of 3.8 mg/ml (the method of Lowry) giving an O.D.₄₄₀ of 1.

References to Table 2.4.

1 Colby and Dalton, 1978

2 Colby and Dalton, 1979

3 This work

the FAD:protein C and Fe_2S_2 :protein C stoichiometries are 0.42-1:1 and 0.41-0.96:1 respectively. In summary, it seems that the FAD: Fe_2S_2 :protein C stoichiometries may not be 1:1:1 as indicated previously, though these differences may well be due at least partially to theoretical decisions rather than differences in the protein C preparations or the initial data. However, the FAD: Fe_2S_2 stoichiometry does appear to be 1:1, in agreement with the conclusions of Colby and Dalton, (1979).

2.3.C. The stability of protein C

Protein C in crude and soluble extracts is extremely unstable in the absence of stabilizing agents such as sodium thioglycollate (see Table 2.5.); Colby and Dalton (1976) initially found only a 0-25% decline in MMO activity

of soluble extracts over 24 hours at 4°C, in the absence of any stabilizing agent, but later reported a 60-90% loss in activity on storage of DEAE-fraction C at 0°C for 20 hours, also in the absence of any stabilizing agent (Colby and Dalton, 1978). Sodium thioglycollate (5 mM) was found to be an effective stabilizing agent since no activity was lost on storage of DEAE-fraction C for 22 hours at 0°C, as compared to the original sample before the addition of thioglycollate (Colby and Dalton, 1978).

Table 2.5. MMO* activity \pm 5 mM thioglycollate.

| sample | thioglycollate | specific activity |
|-----------------|----------------|-------------------|
| crude extract | + | 0.029 |
| crude extract | - | 0.004 |
| soluble extract | + | 0.029 |
| soluble extract | - | 0.001 |

Both extracts were at 20 mg/ml and were prepared at 4°C over a 2 hour period, from a chemostat culture.

* MMO activity was assayed monitoring epoxyethane accumulation from ethene.

Thesedata indicate that in the absence of 5 mM sodium thioglycollate during harvesting and preparation of crude and soluble extracts, protein C undergoes a rapid decay.

The decay of protein C activity in crude and soluble extracts in the absence of stabilizing agents can be variable, but the instability of soluble extracts was

thought to be due to the decay of protein C, since DEAE-fractions A and B retained complete activity on storage at 0°C for 24 hours (Colby and Dalton, 1978). Preparation of soluble extracts in the presence or absence of sodium thioglycollate makes no appreciable difference to the activity of the A and B components of the soluble MMO, although it does stabilize protein C, see Table 2.6.

Table 2.6. The effect of 5 mM thioglycollate on the activity* of proteins A, B, and C in a soluble extract.

| thioglycollate | protein C** addition | specific activity |
|----------------|----------------------|-------------------|
| + | 0 | 0.056 |
| - | 0 | 0.022 |
| + | 0.1 ml | 0.096 |
| + | 0.15 ml | 0.095 |
| - | 0.15 ml | 0.094 |

Extracts were at 42 mg/ml (+) and 48 mg/ml (-) and were prepared from a chemostat culture over a 2 hour period at 4°C, in a collaborative experiment with S.J.Pilkington.

* MMO activity was assayed monitoring epoxypropane accumulation from propene.

** A purified and dilute solution of protein C was added to the assay mixture.

Protein C is not totally stable even in the presence of 5 mM sodium thioglycollate, and appears to decay at very roughly 5% per hour in crude and soluble extracts, and at 2% to 5% per hour in DEAE-fraction C, at 0-4°C, see Table 2.7. These data appear to be in contrast with the previous report that no activity was lost on storage of DEAE-fraction C at 0°C for 22 hours in the presence of 5 mM sodium thioglycollate (Colby and Dalton, 1978).

Table 2.7. Stability of a DEAE-fraction C in the presence of 5 mM thioglycollate.

| incubation time | % activity remaining |
|-----------------|----------------------|
| 0 hours | 100% |
| 3 hours | 85% |
| 5 hours | 75% |
| 6 hours | 66% |
| 17 hours | 0% |

The rate of decay is enhanced at temperatures above 0-4°C, at 17-18°C protein C in DEAE-fraction C decays at very roughly 25% per hour, although protein C is stable to freeze-thawing, for instance no activity was lost on 3 cycles of freeze-thawing with DEAE-fraction C or pure protein C, the maximum number of freeze-thaw cycles to which protein C is subjected. This is consistent with the observation of Colby and Dalton (1976) that no activity was

lost on storage of soluble extracts at -70°C for several months, and protein C does seem to be indefinitely stable at -70°C . However, pure protein C can be more stable or just as stable as protein C in crude and soluble extracts and in DEAE-fraction C, for instance, after 22 hours at 4°C , in the presence of 5 mM sodium thioglycollate, pure protein C retained 70% of its initial activity, and in the absence of any stabilizing agent at $17-18^{\circ}\text{C}$ (sodium thioglycollate was removed from protein C by desalting on Sephadex B-15), protein C retained 38% of its activity over an 8 hour period.

The instability of protein C also appears to show up by other techniques than methane monooxygenase assays; three techniques indicate that protein C undergoes structural alterations in the absence of functional stabilizing agents, namely 5 mM sodium thioglycollate and a temperature of 4°C . Ultracentrifugation was attempted on protein C in the absence of sodium thioglycollate, but otherwise identical conditions to the study in the basic characterization section. Sodium thioglycollate was removed because it has a strong u.v. absorbance and precluded accurate analysis of sedimentation data. The meniscus did not clear of strong u.v. absorbance after 4 hours, with no apparent protein C sedimentation occurring. Visual inspection of the cell, after termination of the run, indicated a yellow-brown zone on the outermost side of the cell, indicative of protein C, and showed that sedimentation of protein C had taken place, but had been obscured in some

way. Therefore it is concluded that in the absence of sodium thioglycollate protein C releases a strongly u.v. absorbing low molecular weight substance (which does not sediment under these conditions), possibly sulphide or some complex of sulphide. The molecular weight estimate by non-denaturing electrophoresis indicated three separate protein C bands, so protein C appears to be present in forms with different electrophoretic properties under non-stabilizing conditions (the absence of thioglycollate and the performance of the experiment at room-temperature), in contrast to the results seen under conditions of functional stabilization, see 2.2.B. Similarly, non-denaturing isoelectric focusing of protein C, in the absence of sodium thioglycollate, for 4 hours at 4°C over a pH range of 3.5-8.5 showed that protein C gave rise to 5 bands with pIs ranging from 4.6-5.2 pH units, with three major bands of which the two most acidic failed to give the Nitro Blue Tetrazolium stain (indicating that these two bands may lack the FAD group, because this prosthetic group appears to be required for reductase activities, see 3.2.). Protein C stabilization by addition of the uncharged reducing agent dithiothreitol was not possible because the gels would not set, and thioglycollate could not be used in these experiments because it is charged. During the running of these gels, separate bands were seen with protein C, and one of these bands was red in colour, as would be expected if the iron-sulphur centre was present on protein C without the yellow-green FAD, for it is the combination of these

three colours that would be expected to produce the yellow-brown colour seen with native protein C. Possible loss of redox centres might explain the multiple bands seen on non-denaturing electrophoresis and isoelectric focusing under conditions where protein C loses its enzymic activities. Possible relationships between redox centre integrity and protein C activity are discussed in 3.2. Redox centre integrity may not be limiting to protein C instability, which might be due to other inactivating effects occurring with protein C, so the putative 40% occupancy of protein C by its redox centres (found by the present author, see 2.3.B.2.) is merely consistent with its observed enzymic instability, and redox centre integrity may not be the cause of protein C instability per se.

CHAPTER 3. The redox centres of Protein C

3.1. Reduction and oxidation of Protein C

Introduction

The redox centres of protein C, namely the FAD and Fe_2S_2 centres, have been examined by reduction and reoxidation with a variety of redox agents, NADH (Colby and Dalton, 1978) NADPH, EDTA/light (Colby and Dalton, 1978), dithionite, O_2 and ferricyanide (also protein A, see 4.3.). It is possible to monitor the state of reduction of protein C spectrophotometrically, because protein C (and both redox centres) undergoes large absorbance changes in the visible region on reduction, as shown in Figure 3.1., and the state of reduction may also be monitored by electron paramagnetic resonance (E.P.R.) spectroscopy, a technique capable of detection and identification of centres with unpaired electrons.

Protein C is amenable to E.P.R. spectroscopy because the Fe_2S_2 centre displays no E.P.R. signal in the oxidized state, and accepts only one electron, when it exhibits a characteristic E.P.R. signal with g-values at 2.047, 1.960, and 1.864 (the g-value is a measure of the degree of orbital restriction of the unpaired electron wavefunction). For the extensively delocalised unpaired electron of the semiquinone, the g-value approaches that of a free electron at $g = 2.0023$; the semi-reduced (semiquinone) form of FAD also has one unpaired electron and exhibits a distinctive E.P.R. signal at $g = 2.002$, in contrast to oxidized FAD (quinone) and the fully reduced 2-electron form of FAD (dihydroquinone), neither of which

Figure 3.1. Spectrophotometric redox titrations of protein C.

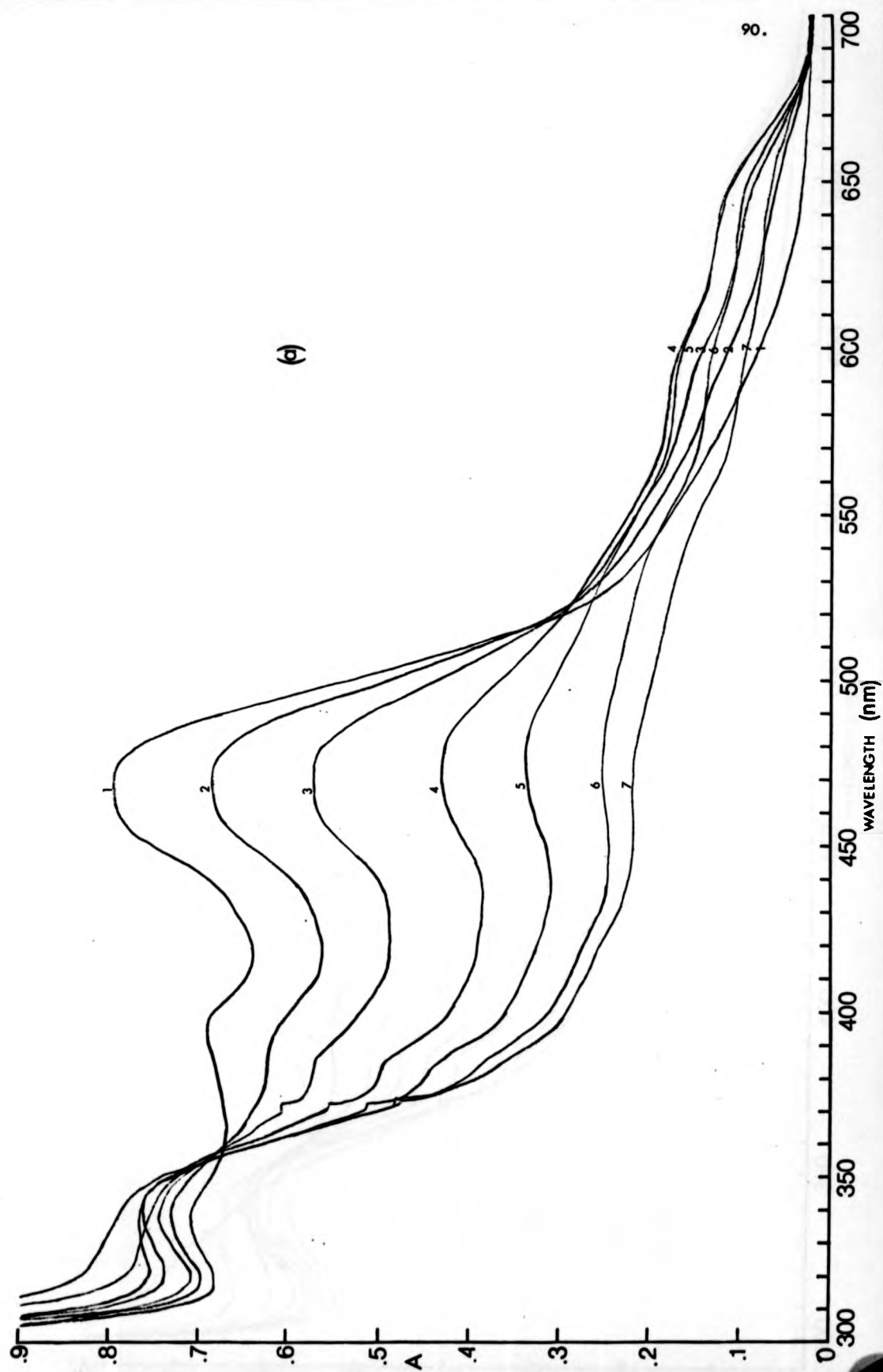
(a) Protein C (141 nmoles in 1.6 ml) was reduced with 0.15, 30, 40, 50, 60, and 75 microlitres of 1.3 mM NADH, (1-7).

(b) Protein C (107 nmoles in 1.42 ml) was reduced with 0.5, 10, 15, 20, 25, 32.5, and 45 microlitres of 1.77 mM NADPH, (1-8). The fully reduced spectrum was obtained by the addition of a buffered dithionite solution, (9).

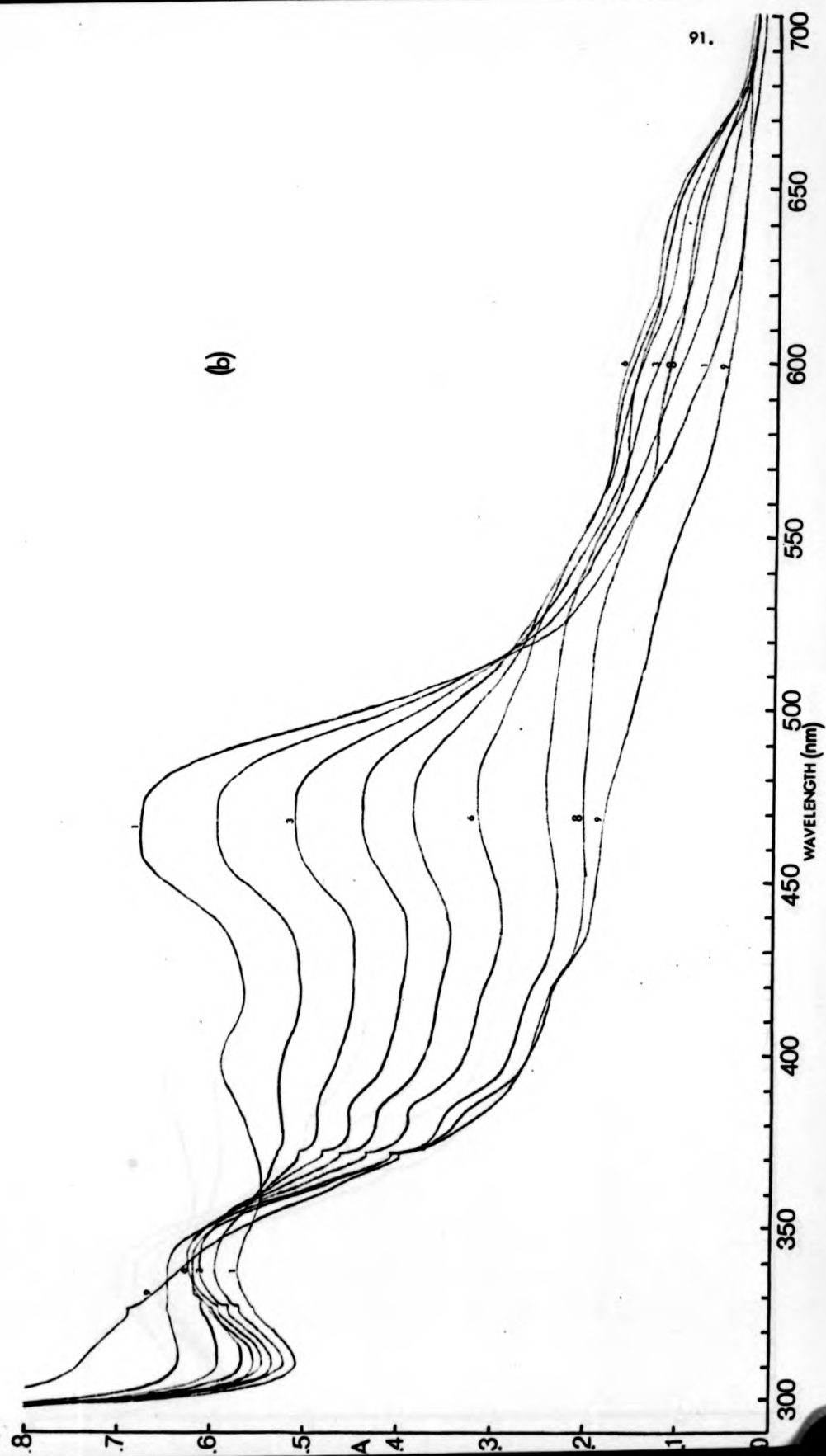
(c) Protein C (147 nmoles in 1.65 ml) was reduced with 0.5, 10, 15, 20, and 30 microlitres of 2 mM dithionite (in 0.25 M Tris-HCl, pH 7.6), (1-6).

(d) Protein C (118 nmoles in 1.65 ml) was reduced with buffered dithionite (1) and reoxidised with 10, 25, 40, 50, and 65 microlitres of 2 mM potassium ferricyanide, (2-6).

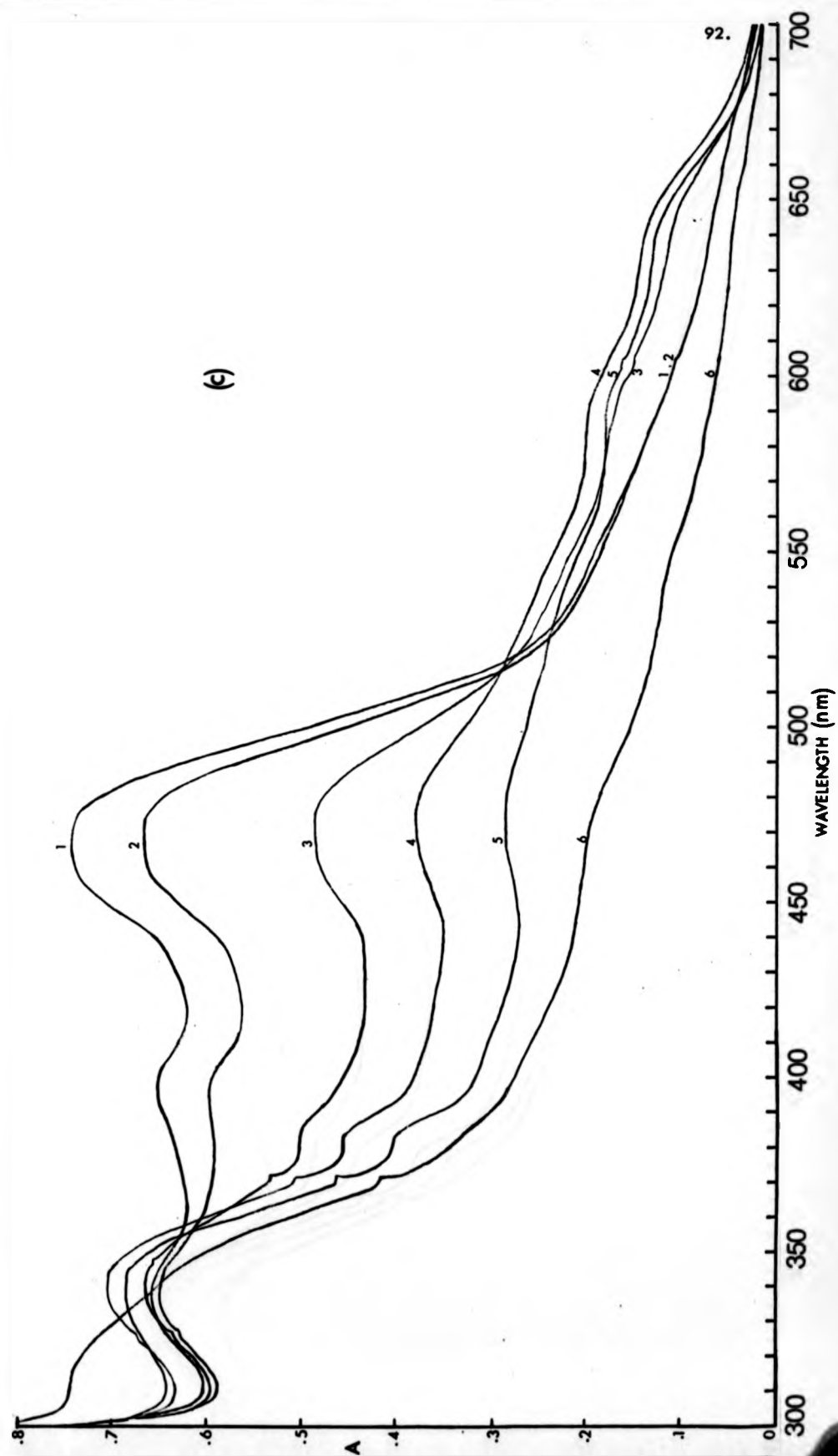
All spectra were obtained at 4°C, in 20 mM Tris-HCl buffer, pH 7.0, in the anaerobic cuvette system detailed in Materials and Methods. The wavelength scans were recorded at 2 nm/sec and a bandwidth of 1 nm. The spectrophotometer was a Pye-Unicam SP8-200 model.

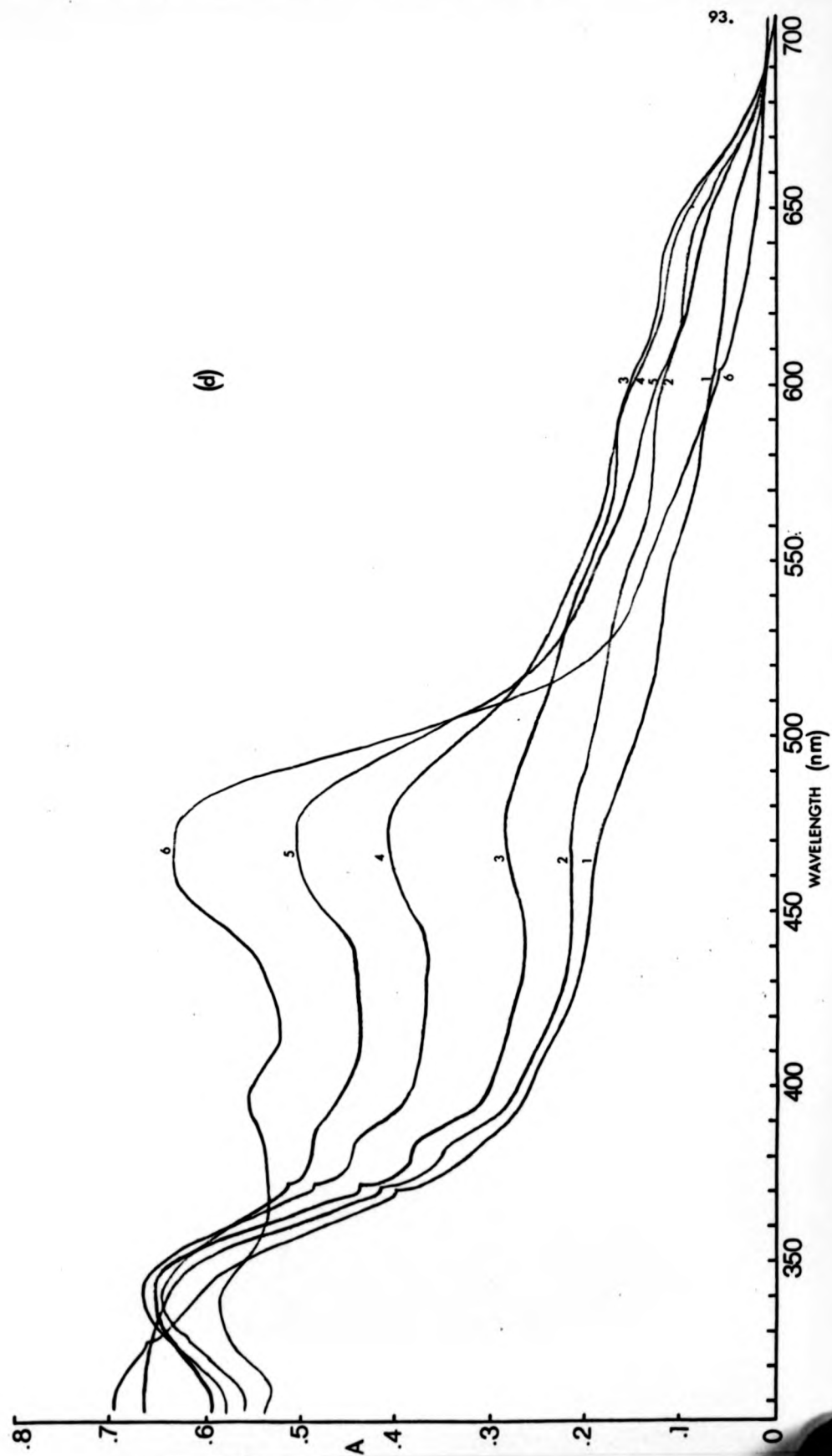


(b)

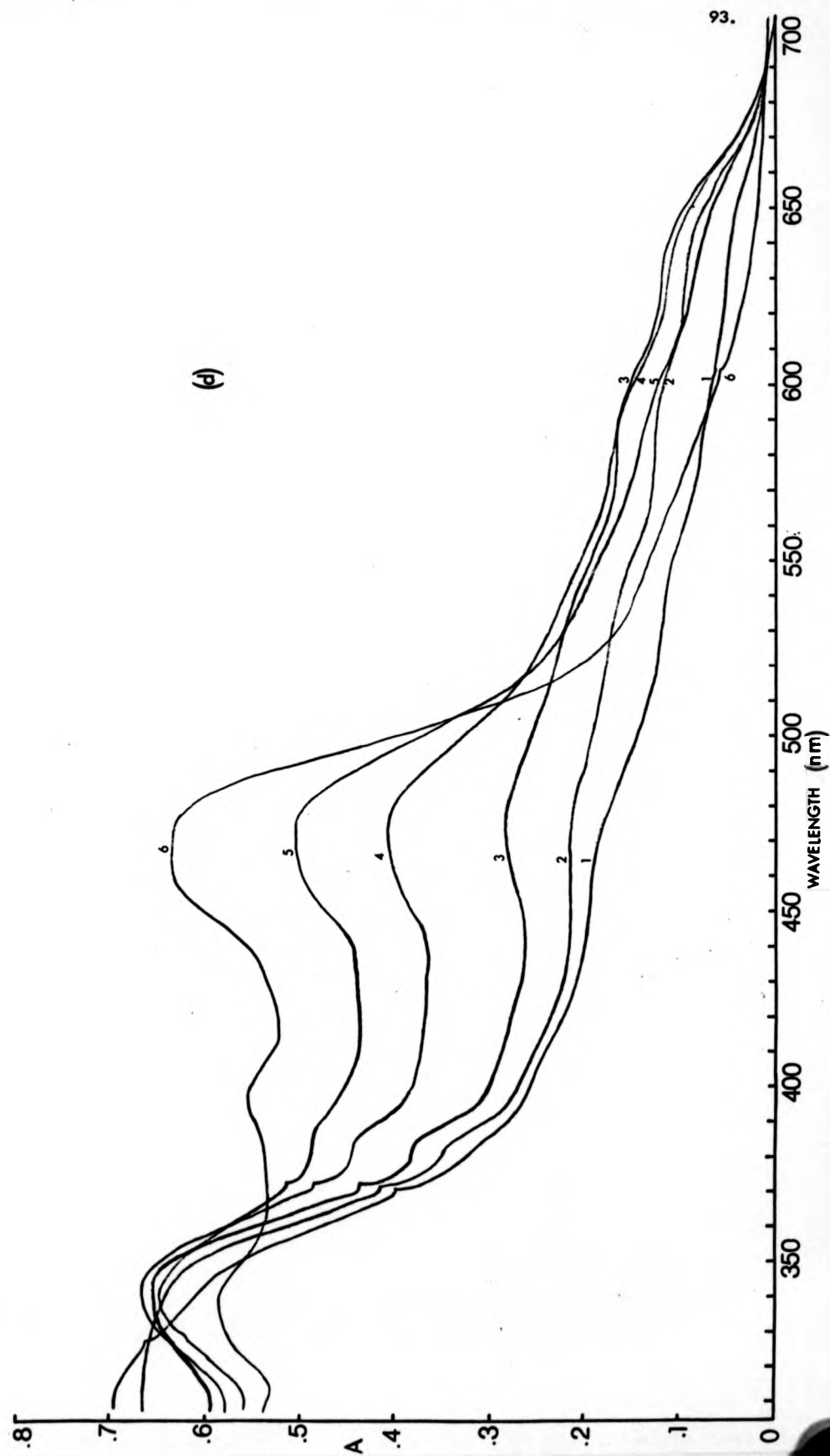


(b)





(d)



(d)

have an E.P.R. signal, since both lack any unpaired electrons, see Figure 3.2. (Colby and Dalton, 1979).

Because protein C and some of the reducing agents react readily with molecular oxygen, anaerobic conditions are necessary to perform quantitative work, and so an anaerobic quartz cuvette apparatus was used. In this apparatus, samples are made anaerobic by serial evacuation and flushing with anaerobic nitrogen, as detailed in Materials and Methods.

Figure 3.2. E.P.R. redox titrations of protein C with NADH.

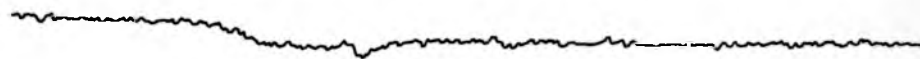
Protein C (a) (414 nmoles in 3.2 ml of 20 mM Tris-HCl pH7.0, at 4°C) was reduced with 10 (b), 15 (c), and 20 (d) microlitres of 11 mM NADH, and samples (0.22 ml) were removed for E.P.R. analysis. Full reduction (e) was effected by the addition of 10 microlitres of about 3 mM dithionite (in 0.25 M Tris-HCl, pH7.6). The experiment was performed using the anaerobic cuvette, detailed in Materials and Methods.

The recording conditions were:- a temperature of 19 ± 1 Kelvin; 1.03 mW microwave power, at a frequency of 9.47 GHz; a modulation of 10 Gauss and a gain of 8×10^4 . N.b. this power level is saturating for the semiquinone signal.

(a)

0 NADH

94a.



(b)

10 NADH

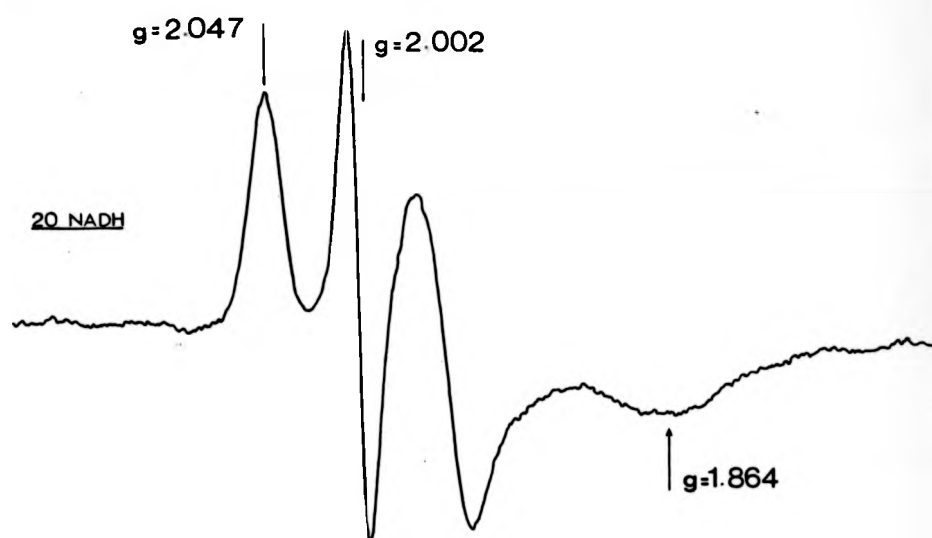


(c)

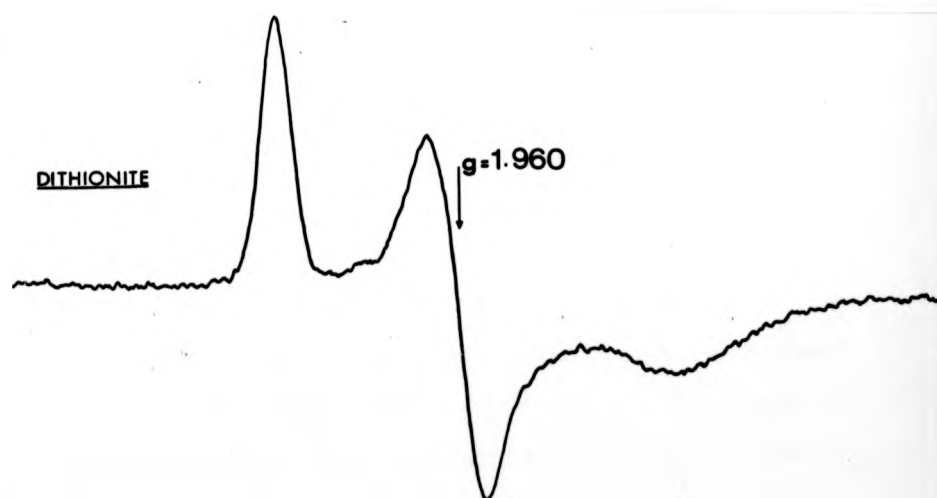
15 NADH



(d)



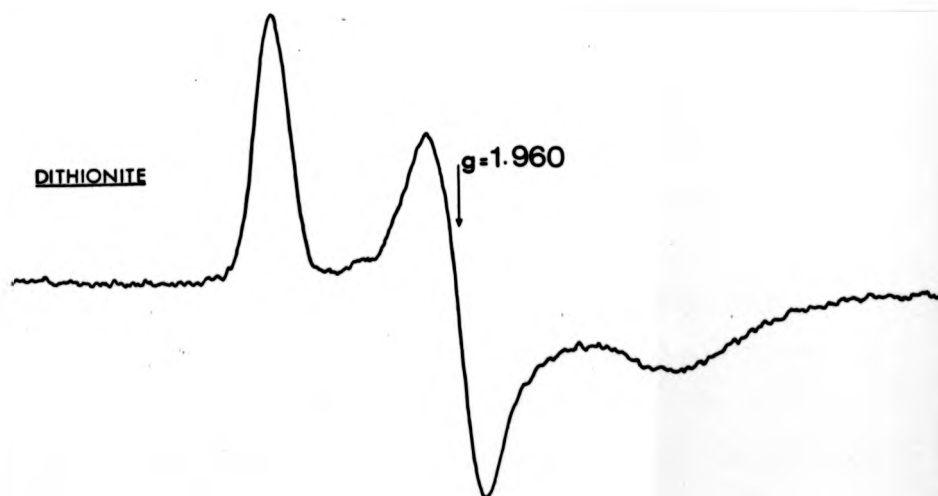
(e)



(d)



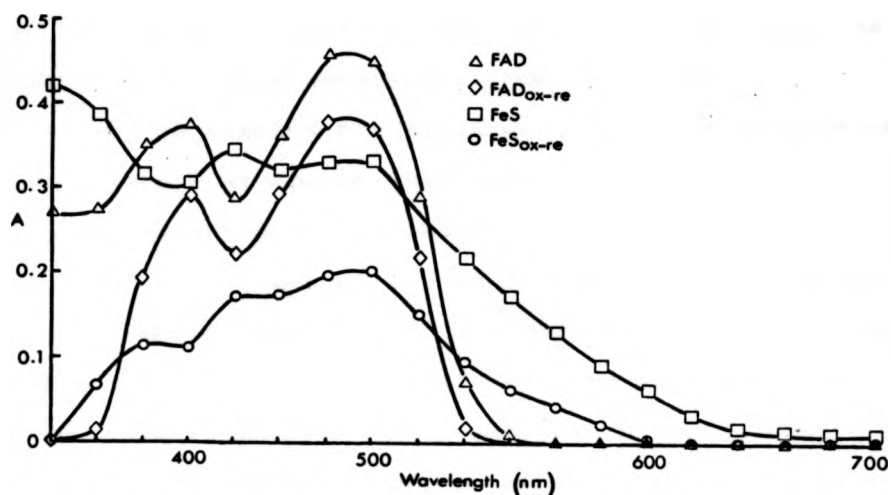
(e)



3.1.A. Resolution of the FAD and Fe_2S_2 redox centre absorbances

Mersalyl is a mercurial agent which reacts covalently with sulphhydryl groups and can be used to destroy the iron-sulphur centre, thereby permitting an evaluation of its contribution to the optical spectrum of protein C. The absorbance change on reduction of the iron-sulphur centre is less than the total absorbance due to this centre (as is also the case for the FAD centre), simplistically because the iron-sulphur centre can only be half-reduced (accepting 1 electron; Orme-Johnson and Beinert, 1969), so retains some absorbance due to the remaining ferric iron (for review see Palmer, 1973). This residual absorbance may be destroyed by treatment of reduced protein C with mersalyl, and subtraction of this absorbance from the fully oxidized protein C iron-sulphur absorbance (determined by treatment of oxidized protein C with mersalyl, see 2.3.B.2.C.) gives the absorbance change due to the iron-sulphur centre on reduction. Subtraction of this latter absorbance change from the total absorbance change of protein C on reduction gives the absorbance change due to the FAD redox centre on reduction. These absorbance changes on reduction may be compared with the overall absorbance due to the FAD and Fe_2S_2 redox centres, which is determined simply by treatment of oxidized protein C with mersalyl, see Figure 3.3. (n.b., mercurials have been used in a similar way to resolve the iron-sulphur absorbance from that of FAD in succinate

Figure 3.3. Resolution of the total and reducible absorbances of the protein C redox centres.



To obtain these data two experiments were carried out, both under anaerobic conditions. Protein C (94 micromolar in 1.1 ml) was either reduced with a slight excess of buffered dithionite, and then treated with mersalyl (1.36 micromole in 0.25 M Tris-HCl pH7.6.), or treated with excess mersalyl and reduced with a slight excess of dithionite. The spectra were compared after a small correction for initial differences in the concentration of the two samples, and the same protein C preparation was used for both experiments. It may be of interest that on mersalyl addition to semi-reduced protein C, some reoxidation of protein C was observed, presumably due to the liberation of ferric ions from the iron-sulphur centre.

dehydrogenase: Davis and Hatefi, 1971; Gutman et al., 1980).

The FAD and iron-sulphur centre of oxidized protein C make large absorbance contributions from 340 nm through to 540 nm, when the FAD absorbance becomes negligible, whereas the iron-sulphur absorbance merely tails off at longer wavelengths and is responsible for the non-zero absorbance of oxidized protein C at longer wavelengths.

The absorbance profiles of the iron-sulphur centre are very similar to those of spinach ferredoxin (Tagawa and Arnon, 1962), which has peaks at 330 nm, 420 nm, and 463 nm, as compared to peaks at 340 nm, 420 nm, and 480 nm for the iron-sulphur centre of protein C, and both proteins have similar ratios for the iron-sulphur centre absorbances at these wavelengths, 1:0.72:0.65 (Tagawa and Arnon, 1962), or 1:0.75:0.77 (Xavier et al., 1981) c.f. 1:0.82:0.79 for protein C. The E.P.R. spectra of these two iron-sulphur centres are also similar, with g-values at 2.05, 1.95, and 1.89 for spinach ferredoxin (for review see Xavier et al., 1981), and g-values at 2.047, 1.960, and 1.864 for protein C (Colby and Dalton, 1979). Similar spectrophotometric behaviour is exhibited by the iron-sulphur centres of both proteins on reduction, when only half the absorbance at visible wavelengths, due to the iron-sulphur centre, is lost; whereas there is very little change in the 300-340 nm region because the lowest energy ligand-Fe(II) bands replace the contribution to the absorbance of the reducible ferric iron (see Palmer, 1973). Not all the absorbance due to the FAD is removed on reduction of protein C (18.6% of

the FAD absorbance at 465 nm remains on reduction of protein C), as compared to reduction of authentic FAD (circa 6%), or with adrenodoxin reductase, for example, where about 11% of the total absorbance due to the FAD at 465 nm remains on reduction (Lambeth and Kamin, 1976).

3.1.B. Spectrophotometric redox titrations of protein C

Protein C was titrated with various redox agents, NAD(P)H, dithionite, and ferricyanide, monitoring reduction by spectrophotometry. The titrations were carried out under anaerobic conditions in Tris-HCl buffer (20 mM, pH 7.0) at 4°C, as detailed in Materials and Methods. The spectra are identical to those seen previously (Colby and Dalton, 1978) and are shown in Figure 3.1. The main features of oxidized protein C are local maxima at 340 nm, 395 nm, and a maximum at 465 nm; oxidized protein C is yellow-brown in colour. On reduction of protein C the peak at 395 nm shifts towards 382 nm, and the 395 nm and 465 nm peaks decrease in unison as reduction proceeds, indicating that they are probably due to the same redox species. On partial reduction of protein C local maxima appear at 340 nm, 520 nm, 580 nm, and 630 nm. The 340 nm local maximum changes in concert with these three shoulders, indicating that these absorbances are due to the same redox species. These local maxima are largest when the 395 nm and the 465 nm peaks are approximately $33 \pm 10\%$ of their oxidized height. At this stage protein C is purple-grey in colour, but as reduction proceeds protein C loses the purple colour and the 340 nm, 520 nm, 580 nm, and

630 nm absorbances fall off together. The redox species causing this behaviour is likely to be a neutral semiquinone (Müller *et al.*, 1972; Colby and Dalton, 1978). The 395 nm peak and the 465 nm peak absorbance decrease on reduction probably involves contributions due to the reduction of oxidized FAD to the semiquinone and dihydroquinone forms, and the iron-sulphur centre of protein C would also be expected to make a significant contribution to the 395 nm and 465 nm peak decreases on reduction, as is the case for succinate dehydrogenase, for example (Davis and Hatefi, 1971). Millimolar extinction coefficients for the various peaks and shoulders are given in Table 3.1., for oxidized and reduced protein C, and for the putative semiquinone form of protein C. The change in extinction coefficient between any two of these redox states may be obtained by subtraction of the appropriate extinction coefficients. Inspection of Figure 3.1. indicates that there is no difference in the protein C spectra on reduction either in the presence of dithionite, NADPH, or NADH, and may indicate that there is no relative shift in mid-point potential of the FAD couples with respect to the Fe_2S_2 couple. However, on reoxidation of reduced protein C absorbance may be lost at wavelengths greater than 340 nm, perhaps due to loss of sulfide from the iron-sulphur centre (*e.g.*, see Figure 4.7.).

Qualitative inspection of the reduction profiles of protein C allows several conclusions to be drawn about the two redox centres. These conclusions from the spectrophotometric data are consistent with the E.P.R. data

Table 3.1. Millimolar* extinction coefficients for protein C.

| wavelength | (mM ⁻¹ cm ⁻¹) | | **E _{s.q.} |
|------------|--------------------------------------|----------------------|---------------------|
| | E _{oxidised} | E _{reduced} | |
| 340 nm | 7.66 | 7.66 | 8.40 |
| 395 nm | 7.67 | 3.97 | 5.19 |
| 465 nm | 8.84 | 1.99 | 4.25 |
| 520 nm | 2.25 | 1.24 | 3.57 |
| 580 nm | 1.0 | 0.40 | 2.25 |
| 630 nm | 0.30 | 0.30 | 1.44 |

* Based on 4.2 mg/ml of protein C giving an O.D.₄₆₅ of 1.0, and a molecular weight of 37,000.

** The extinction coefficients for the "semiquinone" form of protein C, obtained from the absorbances when the semiquinone is maximal, i.e. in semi-reduced protein C. The use of the term "semiquinone form" is not meant to imply that all absorbances are due only to the FAD semiquinone.

(see 3.1.D.) on the relative redox relations of the two redox centres in protein C.

(1) When the semiquinone form of protein C (monitored at 580 nm and 630 nm) is less than half-reduced (to the dihydroquinone, i.e. at a redox potential more positive than the mid-point potential for the semiquinone / dihydroquinone couple), at 465 nm the absorbance is close to the fully

reduced value. Therefore at this stage of protein C reduction the iron-sulphur centre, which makes a strong (reducible) absorbance contribution at this wavelength, has been reduced far beyond the half-reduction stage and is thus at a redox potential more negative than its mid-point potential; similarly the quinone reducible absorbance has also been removed, indicating quantitative removal of the quinone (oxidized) form of FAD, a priori either to the semiquinone or the dihydroquinone, or a mixture of these reduced forms of FAD. It may be concluded that the mid-point potentials for the iron-sulphur centre and for the quinone reduction are greater than the mid-point potential for the semiquinone / hydroquinone couple.

(2) The shift in wavelength of the 395 nm peak of protein C to 382 nm on reduction may also be able to assist in the ordering of the various mid-point potentials. This shift takes place mainly in the initial phase of protein C reduction and is largely complete when the 395 nm and 465 nm peaks have lost half of their reducible absorbance. The reduction spectra of apo-iron-sulphur protein C, which has only the FAD redox centre, (see 3.2.1.) indicate that the wavelength shift may be assigned to the FAD redox centre, and is due to the disappearance of the quinone form of FAD; also the iron-sulphur total and reducible absorbances are both constant over the 380 nm to 400 nm wavelength range. Therefore this shift in wavelength early on in the reduction of protein C, before the maximal semiquinone stage is reached (as indicated by the absorbance at 580 nm and 630

nm). may indicate that the quinone absorbance decreases in preference to the iron-sulphur absorbance, showing that quinone reduction has a more positive mid-point potential than the iron-sulphur mid-point potential. As reduction proceeds to the stage of maximal semiquinone and beyond, the shifted peak maintains its wavelength position, indicating that quantitative conversion of quinone to reduced forms has occurred, but decreases in intensity before the semiquinone absorbance bands decay, putatively due to reduction of the iron-sulphur centre. If the FAD redox centre behaves homogeneously in protein C on reduction (and the stoichiometry for reduction is consistent with this supposition), then since the semiquinone form first appears and then declines as reduction proceeds, this indicates that the quinone / semiquinone couple has a more positive mid-point potential than the semiquinone / dihydroquinone couple, and allows the tentative ordering of the three mid-point potentials as follows:-



(3) These conclusions can explain the behaviour of protein C at 505 to 510 nm on reduction, when a near isosbestic point is seen in the initial stages of reduction (Figure 3.1.). Inspection of the reduction profiles for apo-iron-sulphur protein C shows a similar isosbestic point at 505-510 nm in the initial stages of reduction (Figure 3.14.), probably due to conversion of the quinone to the

semiquinone since the isosbestic point holds until reduction past the maximal semiquinone stage, and then declines, presumably due to decreases in the absorbances due to the quinone and semiquinone forms. The similar behaviour observed in native protein C is consistent with the iron-sulphur centre (which makes a strong absorbance contribution at 505-510 nm), being largely oxidized in the initial stages of protein C reduction and undergoing significant reduction only after the stage where maximal semiquinone is reached. Therefore the observations at 505-510 nm are consistent with the stated order for the three mid-point potentials.

This order would appear to hold independently of the redox agents used here to effect reduction or oxidation, since no qualitative or quantitative changes were seen with NADH, NADPH, dithionite or ferricyanide (a 1-electron acceptor used to reoxidize reduced protein C) or the biological electron acceptor protein A, and indicates that there is no relative potential shift for the three mid-point potentials. The ability to obtain the same redox profiles with the oxidizing agents ferricyanide, oxygen, or protein A, indicates that reduction of the two redox centres is fully reversible on an equilibrium basis.

In summary, spectrophotometric data indicates that on reduction of protein C under equilibrium conditions, semiquinone formation precedes iron-sulphur centre reduction, which in turn precedes the formation of dihydroquinone, and the reverse sequence holds for oxidation of protein C.

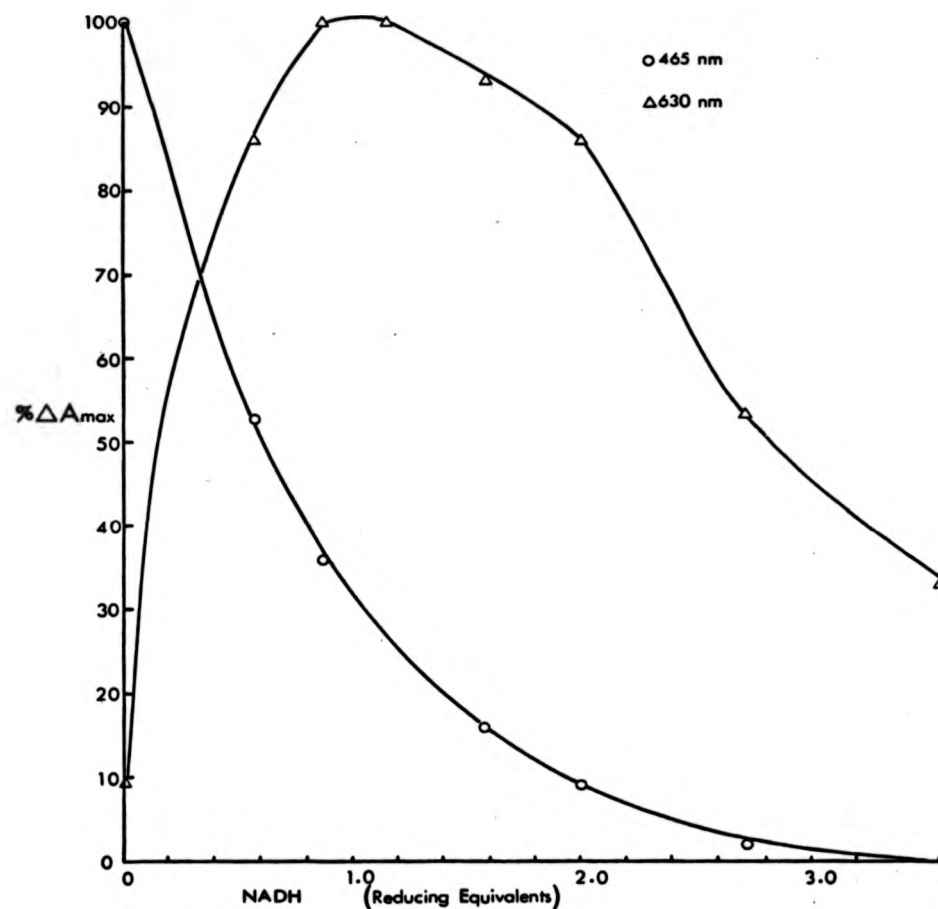
3.1.B.2. Stoichiometries of protein C for redox equivalents

The stoichiometries for anaerobic spectrophotometric titration of protein C (100-200 nmoles in 1.2-2 ml) with the redox agents NADH, NADPH, dithionite, and ferricyanide (with reduced protein C) indicate that protein C accepts 1.2 ± 0.4 electrons (mean of 9 titrations) on a polypeptide basis, using a molecular weight of 37,000 and an $O.D_{440} = 1.0$ being given by a protein concentration of 4.2 mg/ml (see Materials and Methods). The previous estimate indicated that protein C would accept 2.4 electrons per polypeptide, using a molecular weight estimate of 42,000 and an $O.D_{440} = 1.0$ being given by a protein concentration of 3.8 mg/ml (Colby and Dalton, 1978), results in a stoichiometry of 1.9 electrons per protein C polypeptide with present estimates of the protein concentration. Using redox centre absorbances to estimate the redox centre concentration ($E_{440} \text{ FAD} = 11.3 \times 10^3$, $E_{440} \text{ Fe}_2\text{S}_2 = 9.9 \times 10^3$, see 2.3.B(2).), present work indicates that each FAD/iron-sulphur pair accepts (or donates) 2.9 ± 1.0 electrons, which is consistent with the ability of FAD to accept 2 electrons and the ability of the iron-sulphur centre to accept 1 electron. However, the present estimate of the previous stoichiometry (Colby and Dalton, 1978) is inconsistent with the concentration of redox centres present, estimated on an absorbance basis, and would require approximately 1.6 times the concentration of redox centres to agree with the stated stoichiometry. The most obvious interpretation of present data is that there are protein C polypeptides without redox centres, since if each C

polypeptide had 1 FAD (capable of accepting 2 electrons) and one Fe_2S_2 centre (able to accept 1 electron) a stoichiometry of 3 electrons per protein C molecule would be expected. The stoichiometry is consistent with the redox centre concentration on an absorbance basis, and so is also consistent with the estimated 42% occupancy of protein C with regard to redox centres (see 2.3.B(2)). Redox centres might be lost during the DEAE-cellulose step used to produce fraction C.

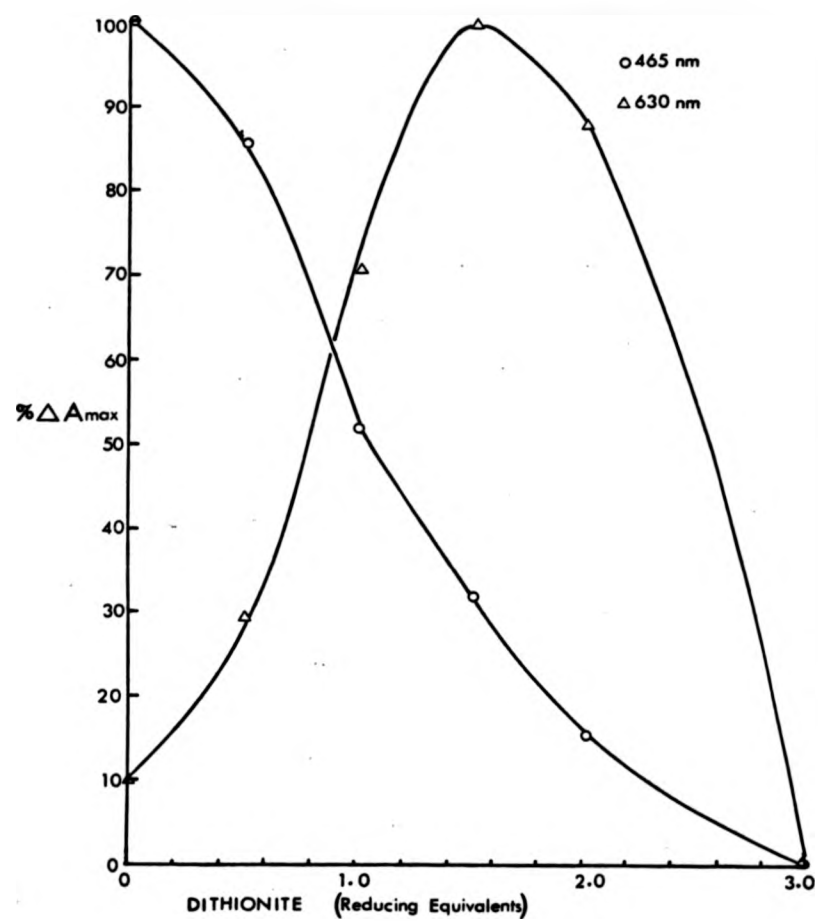
It is possible to correlate the course of reduction with the reducing equivalents added, see Figure 3.4. After 1 electron has been added, the semiquinone absorbance is 70% to 100% of the maximum, as indicated by the 630 nm and 580 nm absorbance peaks. A second electron causes only slight removal of the semiquinone, but results in most of the reducible absorbance at 465 nm being removed, presumably due to reduction of the iron-sulphur centre. A third electron then causes substantial removal of the semiquinone form. The correlation of the 1-electron form of protein C with the maximal semiquinone form indicates that the quinone is initially reduced mainly to the semiquinone form, e.g. appreciable conversion of the quinone to the dihydroquinone could not account for this result. Confirmation of this conclusion comes from estimation of the millimolar extinction coefficient of the semiquinone absorbance at 580 nm (after a small correction for the iron-sulphur centre absorbance) on a redox centre concentration basis, at $E_{580} = 3.6 \text{ (mM}^{-1} \text{ cm}^{-1})$. This value is close to extinction coefficients for other neutral

Figure 3.4. The titration of protein C redox centres with reducing equivalents.



(a) Protein C (155 nmoles in 1.5 ml) was reduced with 1 mM NADH in the anaerobic cuvette. Electron equivalents of the reducing agent added were estimated on a redox centre basis.

With NADH, residual semiquinone is present at the end of the titration, perhaps due to the couple produced by NADH and NAD^+ .



(b) Protein C (147 nmoles in 1.65 ml) was reduced with 2 mM dithionite in the anaerobic cuvette.

semiquinones e.g. 3.4₀₀₀ to 3.9₄₀₀ for the neutral semiquinone form of Azotobacter flavoprotein, a typical neutral semiquinone (Massey and Palmer, 1966), and is therefore consistent with quantitative conversion of the quinone to the semiquinone, as opposed to the dihydroquinone, when a decreased extinction coefficient might be expected. Similarly, quantitative removal of the absorbance at 465 nm in the 2-electron form of protein C indicates that the second electron is used mainly to reduce the iron-sulphur centre of protein C, supported by the semiquinone only being substantially converted to the dihydroquinone after the reduction of the iron-sulphur centre.

In summary, the 1, 2, and 3 electron forms of protein C appear to be the oxidized iron-sulphur / semiquinone form, the reduced iron-sulphur / semiquinone form, and the reduced iron-sulphur / dihydroquinone form respectively, so that the quinone is converted first to the semiquinone, and only then to the dihydroquinone, as reduction proceeds, with the iron-sulphur centre being a reduced intermediate between these two reduced forms of FAD.

Therefore the redox centres of protein C appear to behave homogeneously with respect to the order of reduction, and thus to the relative mid-point potentials exhibited by the three redox couples in protein C.

3.1.C. E.P.R. redox titrations of protein C

The E.P.R. method was used to attempt a resolution of the FAD and Fe-S₂ centres of protein C, and thence to

investigate the relative redox relations of the two redox centres. Spectra were obtained by Dr. D.J. Lowe at the University of Sussex with a Bruker spectrometer, at 19 K (with a helium cryostat, Oxford instruments) since the iron-sulphur centre of protein C is only observable by E.P.R. at low temperature, when less relaxation occurs due to decreased thermal vibration. Initially, simple aerobic E.P.R. redox titrations of protein C were carried out with around 30 nmoles of protein C in different redox states in 20 mM Tris-HCl buffer, pH 7 (4°C), whereas previous work was done in 50 mM Tris-HCl at pH 7 (Colby and Dalton, 1979), using NADH or dithionite as reducing agents. The sample (0.2 ml) was placed into quartz E.P.R. tubes (3 mm internal diameter) and frozen in liquid nitrogen to record the E.P.R. spectrum. Protein C gave very similar E.P.R. spectra to those seen in previous work (Colby and Dalton, 1979, also see Figure 3.2.), except for a slightly smoother iron-sulphur differential spectrum and no rhombic iron at $g = 4.3$, indicative of high spin ferric iron. Other than the free-radical signal, assigned to the semiquinone (Colby and Dalton, 1979), and the iron-sulphur centre signal assigned to an Fe_2S_2 centre, no other E.P.R.-active species were seen, indicating that protein C seems to be pure by E.P.R. standards. The iron-sulphur centres of protein C (Colby and Dalton, 1979) and Spinach ferredoxin both have rhombic symmetry (less than axial symmetry, where $g_x = g_y \neq g_z$, e.g. adrenodoxin). Oxidized protein C has no E.P.R.-active centres (see Figure 3.2.), since the quinone and the oxidized iron-sulphur centre have no unpaired electrons. As

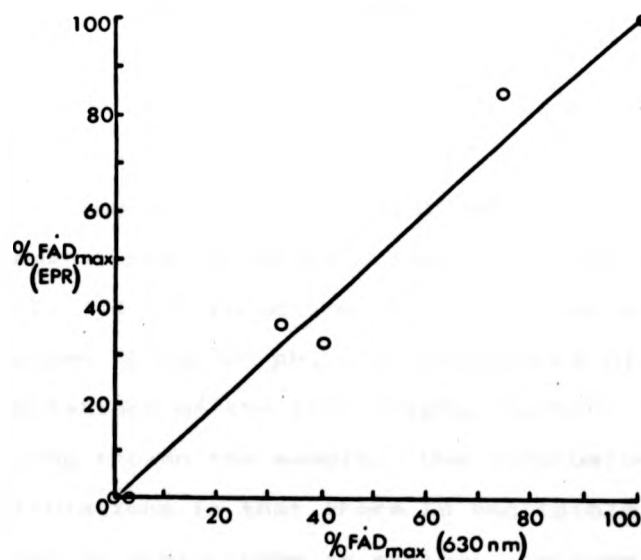
reduction with either NADH or dithionite proceeds, the semiquinone signal due to the FAD redox centre and then the reduced iron-sulphur centre both increase, and with further reduction the semiquinone signal decays, due to concomitant dihydroquinone production, while the iron-sulphur signal intensity remains. Quantitative E.P.R. / optical titrations were carried out with protein C to see if there was a correlation between the semiquinone levels detected by the two techniques, since by this method it may be possible to correlate the degree of iron-sulphur centre reduction with the level of semiquinone, on reduction of protein C. Protein C (414 nmoles in 3.2 ml of 20 mM Tris-HCl pH 7, 4°C) was reduced anaerobically with NADH in the anaerobic cuvette (see Materials and Methods), with reduction monitored spectrophotometrically, and samples (0.2 ml) were transferred by means of gas-tight syringes to suba sealed E.P.R. tubes made anaerobic by repeated evacuation and flushing with anaerobic nitrogen (see Materials and Methods). Samples were frozen and stored under liquid nitrogen for transportation to the University of Sussex, where the spectra were obtained.

Spectra were recorded at 9.46 GHz and a modulation of 10 gauss. The semiquinone signal was recorded for quantitation at a much lower microwave power (which may have been 1.03 microwatts) than the iron-sulphur signal (1.03 milliwatts), and both signals were recorded under non-saturating conditions, the semiquinone at a gain of 2×10^5 and the iron-sulphur at a gain of 8×10^4 . The signals were quantitated by taking the peak height of the

differential E.P.R. spectra, and were corrected for minor deviations from 19 Kelvin by assuming that signal intensity is inversely proportional to the temperature. The $g = 2.047$ iron-sulphur signal was used for quantitation because it is less interfered with (by the semiquinone signal) than the $g = 1.960$ signal and more intense than the $g = 1.864$ signal.

The level of semiquinone indicated by the absorbance bands at 580 nm and 630 nm (see Figure 3.5.) paralleled the level of semiquinone by E.P.R., indicating that the semiquinone had not undergone a large redox shift near liquid helium temperatures (19 Kelvin).

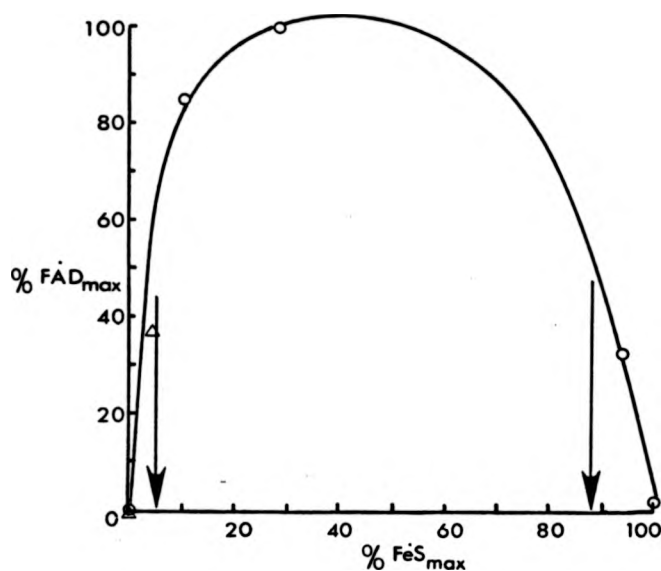
Figure 3.5. Correlation of the level of semiquinone by E.P.R. and optical analysis.



The extent of reduction of the iron-sulphur centre may be correlated with the amount of semiquinone present, see Figure 3.6. Qualitatively, this result indicates that the order of reduction for the protein C redox centres is semiquinone formation, followed by iron-sulphur reduction, followed by dihydroquinone production. Therefore this result agrees with inferences made from spectrophotometric redox titrations of protein C (3.1.B.), and would allow a reasonable estimate of the mid-point potential of the iron-sulphur centre if the semiquinone mid-point potentials were known. These latter mid-point potentials have been estimated spectrophotometrically in the presence of redox dyes, since the semiquinone absorbance bands at 580 nm and 630 nm clearly resolve from quinone and the iron-sulphur contributions (see 3.1.D.). The optical redox contribution of the iron-sulphur centre may be calculated at any stage of reduction knowing its fully oxidized contribution and by assuming that the extent of reduction as measured by E.P.R. reflects the extent of iron-sulphur reduction measured optically (Olson *et al.*, 1974). Since the E.P.R. experiments are conducted at near liquid helium temperatures, possible drawbacks to the method are changes in E_m (if the reduction is accompanied an enthalpy change), changes in buffer pH, and disturbance of the spin populations of the iron-sulphur centre, in addition to having frozen the sample. One conclusion of these calculations is that there is negligible quinone when the level of semiquinone is maximal, because, *e.g.* at 465 nm the

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Figure 3.6. Relative redox relations of the flavin and iron-sulphur centre in protein C by EPR.



Conditions were those described in Figure 3.2. The open circles correspond to reduction of protein C with NADH, and dithionite (10 microlitres of around 3 mM dithionite in 0.25 M Tris-HCl, pH7.6) to achieve full reduction of protein C. The open triangles correspond to reoxidation of the NADH-reduced protein C with 10 and 15 microlitres of 20 mM ferricyanide. The arrows indicate the % reduction of the iron-sulphur centre at the redox potentials corresponding to the mid-point potentials of the FAD redox centre.

iron-sulphur centre absorbance accounts for nearly all the remaining reducible absorbance when the level of semiquinone is maximal. An observation on the saturation of the semiquinone by Dr. D.J.Lowe suggests that the semiquinone is more relaxed when the iron-sulphur centre is reduced, indicating that the two redox centres are within 50 Å of each other. When the iron-sulphur centre was 10% reduced, under non-saturating power conditions (1.03 microwatts) about 2.2 times as much semiquinone was present as when the iron-sulphur centre was 94% reduced, but at 1.03 milliwatts, the power level at which the iron-sulphur centre was examined, both semiquinone signals had similar intensities, indicating superior relaxation of the semiquinone in the presence of a reduced iron-sulphur centre.

The ability of protein C to be reduced to the 1, 2, or 3 electron stage by NADH indicates that protein C can possess an odd number of electrons, so protein C molecules may be interacting, and are stable with 1, 2, or 3 electrons. This is consistent with a role for protein C of splitting up high potential electron pairs from NADH for transfer to protein A. The first electron promotes semiquinone formation, the second electron reduces the iron-sulphur centre, and the third electron causes reduction of the semiquinone to the dihydroquinone. The E.P.R. evidence therefore agrees with the optical data, despite reservations as to temperature induced alterations of the iron-sulphur centre E.P.R. signal, for instance.

3.1.D. Titration of protein C in the presence of redox dyes

Redox dyes can serve as a probe of the redox atmosphere, which may be shifted by the addition of a reducing agent such as dithionite or NADH, or an oxidizing agent such as ferricyanide. The redox potential will dictate the degree of reduction or oxidation of the redox dye, and the extent of reduction or oxidation may be found by spectrophotometry if the dye undergoes a colour change such as bleaching. The redox potential E , is then given by the Nernst equation, if the mid-point potential, E_m (the redox potential at which the dye is half reduced, when $E = E_m$), of the dye is known:-

$$E = E_m - RT/nF \times \ln[\text{dye}_{\text{reduced}}]/[\text{dye}_{\text{oxidized}}]$$

R = gas constant; T = temperature in Kelvin; F = Faraday constant; n = number of electrons transferred.

If the redox dye were in equilibrium with protein C, then the mid-point potentials of the various protein C peaks and redox centres might be estimated by titration of protein C in the presence of an appropriate redox dye. The extent of reduction of the dye would indicate the redox potential of the sample, via the Nernst equation, and the reduction of the redox centres of protein C could be correlated with the redox potential to yield the three mid-point potentials, that for the quinone / semiquinone couple, for the semiquinone / dihydroquinone couple, and for the oxidized and reduced iron-sulphur centre.

Anaerobic spectrophotometric redox titrations were performed on protein C (100 to 150 nmoles in around 2 ml of 20 mM Tris-HCl pH7. 4°C. with 5 mM sodium thioglycollate) using the anaerobic cuvette system detailed in Materials and Methods, in the presence of a small amount (15-20 nmoles) of Safranin O ($E_m = -268$ mV) or Nile Blue A ($E_m = -147$ mV) (At 283 K and pH7.5; Walker and Mortenson, 1973), both of which accept two electrons to become bleached. The redox potential range covered by these two dyes seemed a likely choice because the redox centres in protein C may be strongly reduced by NAD(P)H, which has a mid-point potential of -320 mV, and should therefore have a more positive mid-point potential than -320 mV. Addition of either dye to protein C gave a spectrum which was the sum of the individual absorbances, so the Beer-Lambert law holds for these systems. Both dyes are substrates for protein C, being readily decolourised on the addition of NADH in the presence of catalytic amounts of protein C, but not with NADH alone, so would be expected to equilibrate readily with protein C under the conditions holding for these experiments, as was indicated by the stability of the spectra in between the additions of titrant. Protein C was reduced with dithionite or NADH and reoxidized with ferricyanide, both in the presence of the two separate redox dyes. Qualitatively, protein C is reduced before Safranin O (Figure 3.7.), but after Nile Blue A (Figure 3.8.), indicating that the mid-point potentials of protein C will be within this range of redox potentials, i.e. -268 to -147 mV.

Figure 3.7. Titration of protein C and Safranin O with dithionite.

(a) The reduction of protein C relative to Safranin O allows estimation of the mid-point potential for the semiquinone / dihydroquinone couple, since at the mid-point for this couple (monitored at 630 nm), the dye was around 70% oxidised. The data was taken from (b).

(b) Protein C (146 nmoles in 2.14 ml 20 mM Tris-HCl pH7.0, 4°C) was titrated in the anaerobic cuvette in the presence of 19.5 nmoles of Safranin O, adding 10, 15, 20, 25, 30, 35, and 50 microlitres of 3.5 mM dithionite (in 0.25 M Tris-HCl pH7.6).

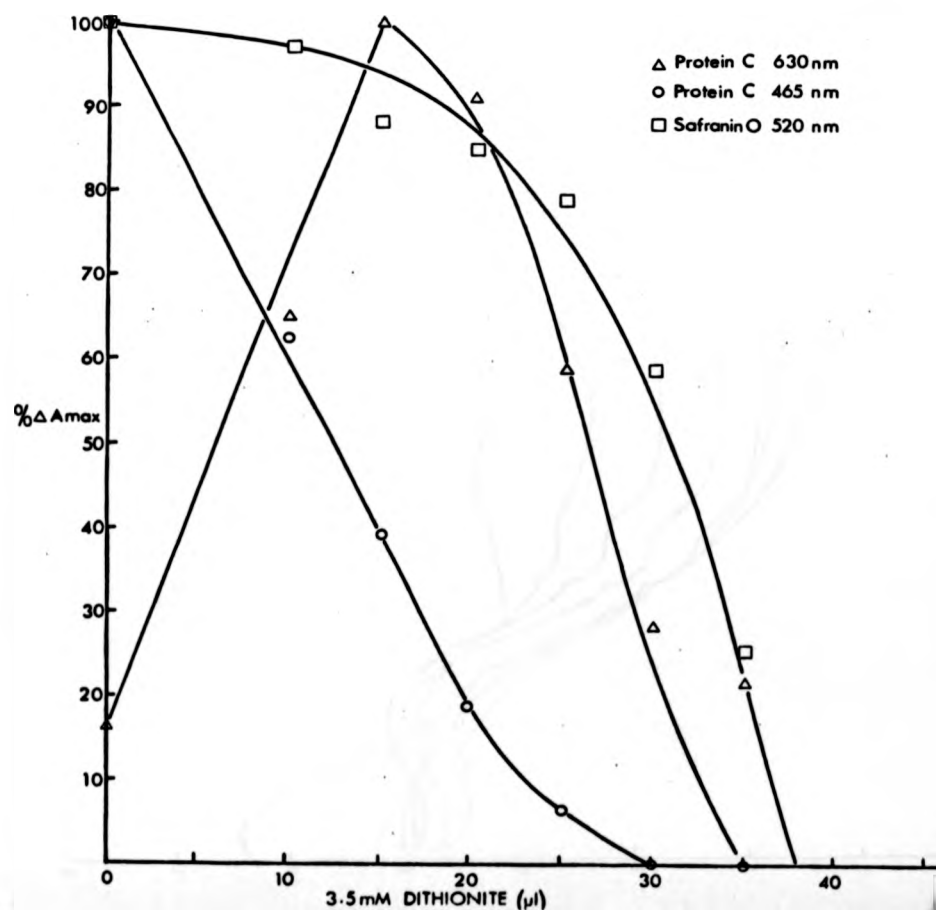
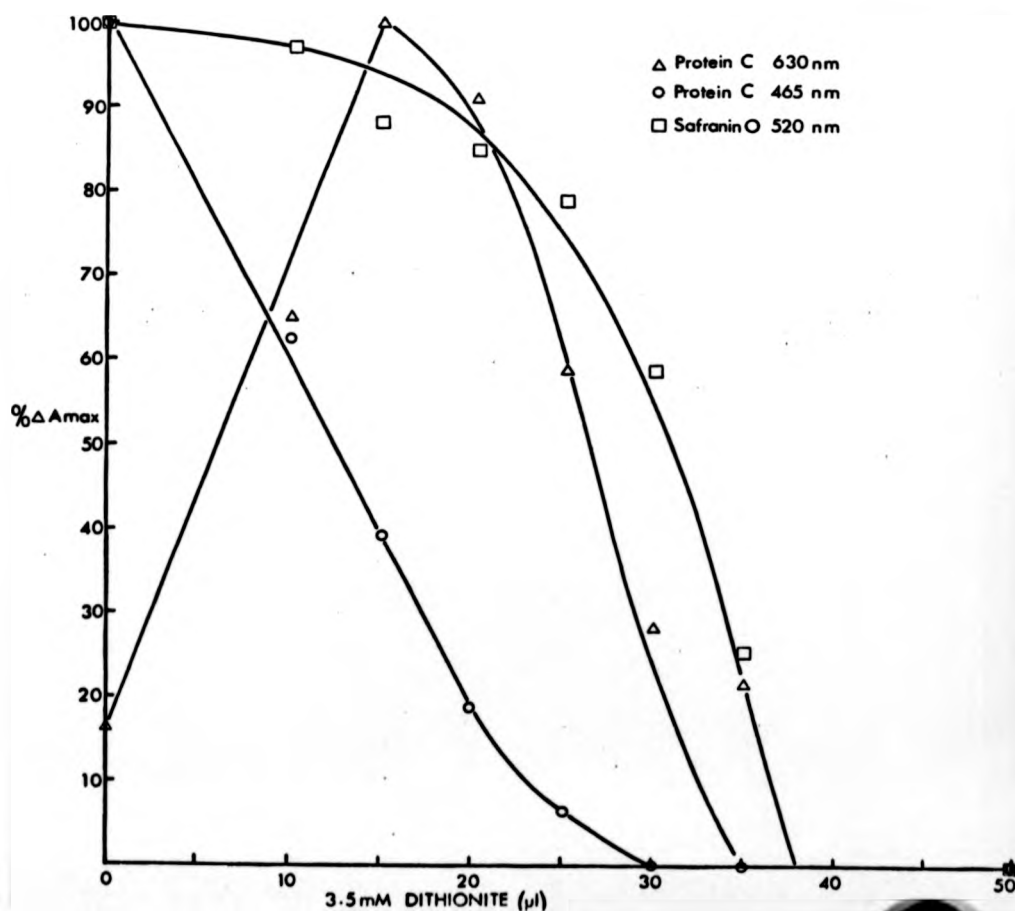


Figure 3.7. Titration of protein C and Safranin O with dithionite.

(a) The reduction of protein C relative to Safranin O allows estimation of the mid-point potential for the semiquinone / dihydroquinone couple, since at the mid-point for this couple (monitored at 630 nm), the dye was around 70% oxidised. The data was taken from (b).

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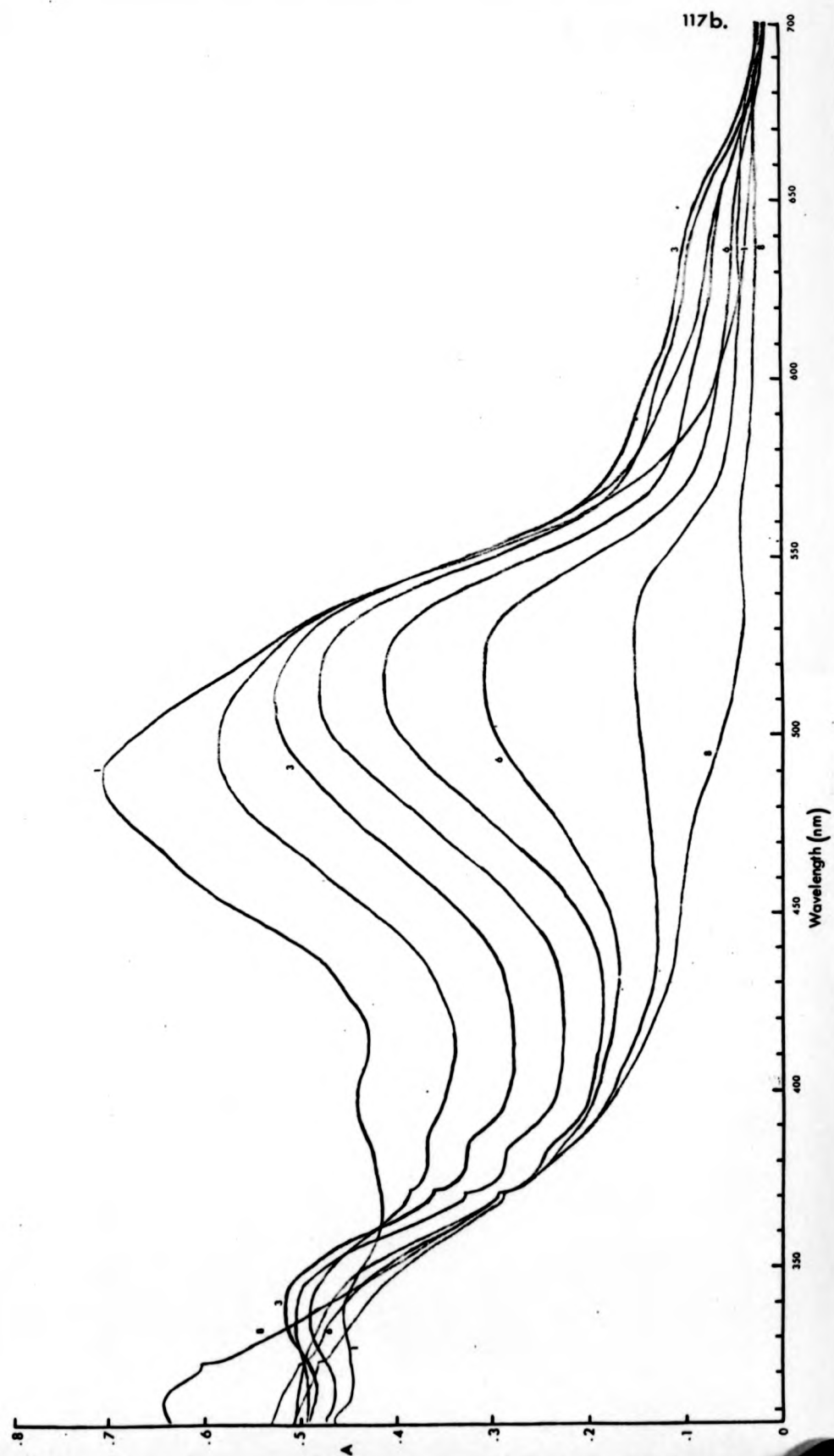
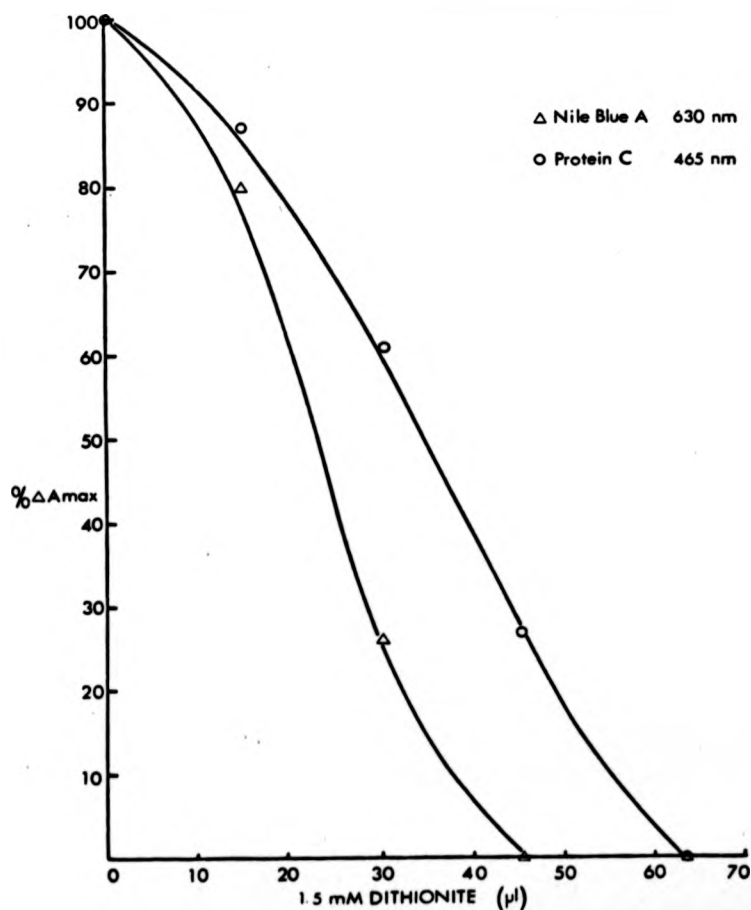
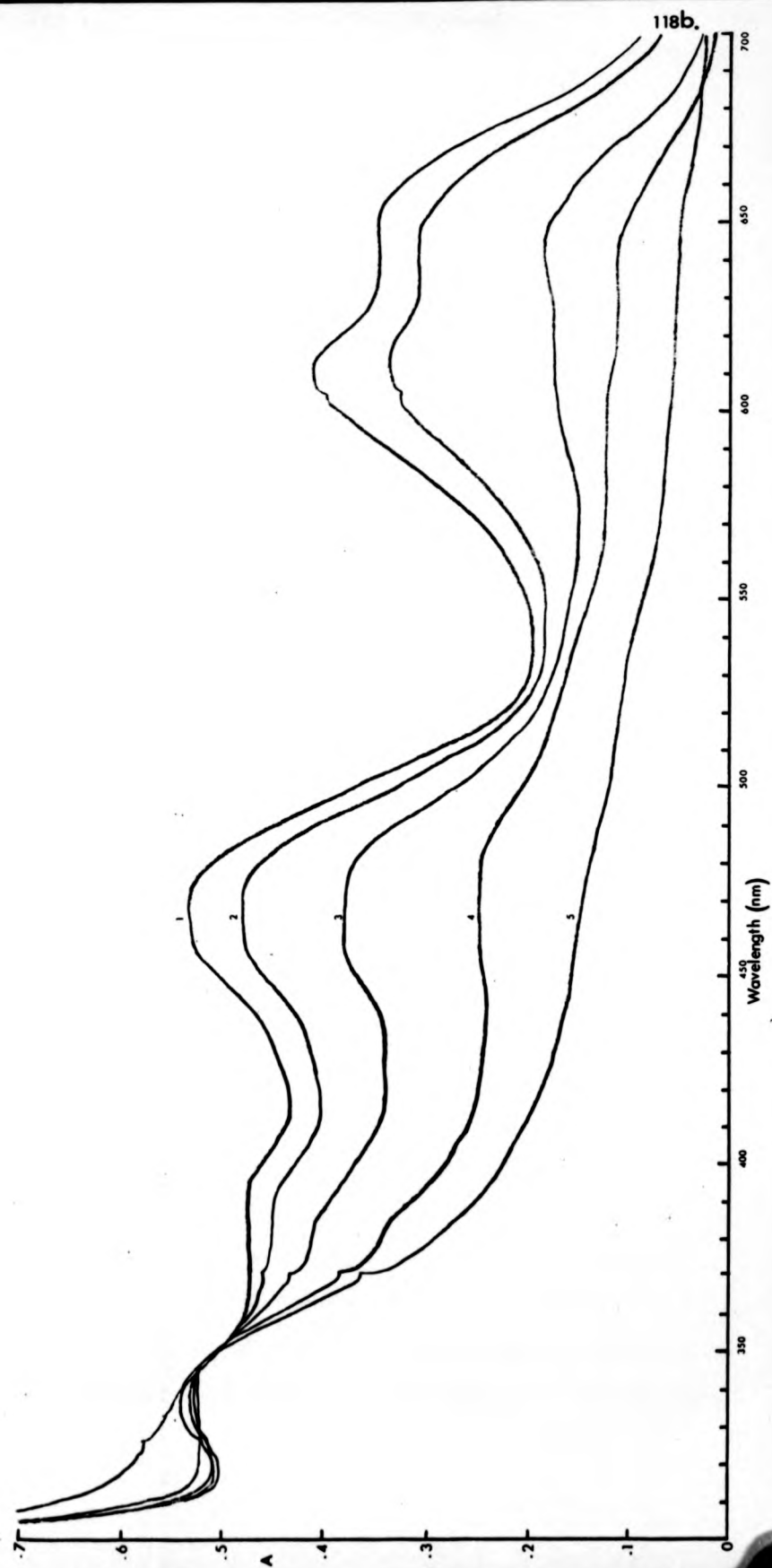


Figure 3.8. Titration of protein C and Nile Blue A with dithionite.

(a) The reduction of protein C relative to Nile Blue A allows estimation of the quinone / semiquinone couple, knowing the relationship between the absorbance changes of protein C at 465 nm and at 630 nm (assigned to the semiquinone). At the mid-point of the 630 nm absorbance (when the 465 nm absorbance is about 75% oxidised), the dye was around 53% oxidised. The data was taken from (b).

(b) Protein C (112 nmoles in 2 ml 20 mM Tris-HCl pH7.0, 4°C) was titrated in the anaerobic cuvette in the presence of Nile Blue A (O.D.₄₃₀ = 0.3), adding 15, 30, 45, and 75 microlitres of 1.5 mM dithionite (in 0.25 M Tris-HCl pH7.6).





Safranin O has negligible absorbance at 630 nm, so the semiquinone absorbance band may be monitored at this wavelength, and also Safranin O has an isosbestic point for reduction at 406 nm, so protein C absorbance changes on reduction may be followed here (which are related linearly to the reducible absorbance at 465 nm). This allows the protein C absorbance to be estimated at 520 nm (knowing the relations between these various protein C peaks on reduction) where Safranin O absorbs, and thus the Safranin O absorbance changes on reduction may be found accurately. Safranin O undergoes considerable reduction by the stage of the redox titration where the semiquinone is half reduced to the dihydroquinone, and this allows accurate estimation of the mid-point potential for this redox couple at -260 ± 5 mV. At this stage of reduction, the removal of the reducible absorbance at 465 nm indicates that both the iron-sulphur couple and the quinone / semiquinone couple are less than the mid-point potential of the dye.

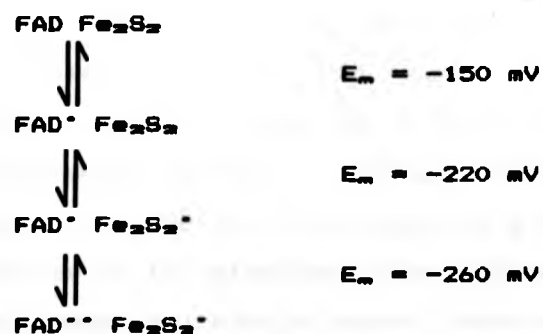
The quinone / semiquinone couple was too positive to be estimated accurately with safranin O, and so Nile Blue A was used to ascertain this mid-point potential, at -150 ± 20 mV. Qualitatively, by the maximal semiquinone stage there is no oxidized dye left, indicating that the iron-sulphur couple and the semiquinone / dihydroquinone couples have more negative mid-point potentials than -147 mV. Nile Blue A has an isosbestic point for reduction at 465 nm, so the absorbance changes at 465 nm on reduction may be used as an index of the degree of semiquinone formation

(only quinone and iron-sulphur absorbances contribute at this wavelength. see 3.1.B(1).), and these values may be subtracted to give an accurate estimate of the absorbance at 630 nm due to Nile Blue A on reduction. The iron-sulphur E_m may be estimated by reference to the semiquinone mid-point potentials and the E.P.R. data in Figure 3.6., assuming $n = 1$ for the iron-sulphur centre (a priori a reasonable assumption since this centre accepts or donates only one electron), at -220 ± 20 mV. The iron-sulphur E_m is consistent with the range quoted by Xavier et al. (1981) at -220 mV to -440 mV.

Oxidation profiles for both redox dyes gave similar results to those for reduction, supporting the conclusion that protein C and the dyes are in equilibrium. No difference was seen in the redox profiles with or without stoichiometric NADH (used instead of dithionite), indicating that NADH does not cause any appreciable shift in the mid-point potentials of the three protein C couples. This observation is consistent with the previous observation that protein C absorbance redox profiles are independent of the redox agent used (see 3.1.B.). NAD^+ binds protein C loosely, as indicated by a K_2 of 5 mM for inhibition of the NADH-acceptor reductase reaction (see 4.2.B.), so might not be expected to interact with protein C in these experiments, where it is present in a slight stoichiometric excess at best. This result is in contrast to that found for adrenodoxin reductase, for example, where $NADP^+$ shifts the quinone / dihydroquinone couple by approximately 100 mV

(Lambeth and Kamin, 1976). These mid-point potentials are consistent with the qualitative observations on protein C made earlier in this Chapter. The mid-point potentials of protein A are very approximately +150 mV and -150 mV, so the estimated mid-point potentials of protein C are consistent with the ability of protein C to pass electrons onto protein A from NADH ($E_m = -320$ mV), under anaerobic conditions (Colby *et al.*, 1979; Dalton, 1980; see 4.3.A.), and it may be predicted that protein A should be capable of major oxidation of protein C under equilibrium conditions (see 4.3.A.). The mid-point potential data is consistent with a role for protein C of catalyzing electron transfer from NADH to protein A, possibly in 1 electron steps, since protein C can support 1, 2, or 3 electrons (at least under equilibrium conditions, see Figure 3.9.), acting to split electron pairs from NADH (see 1.3.A.).

Figure 3.9. Redox states of the iron-sulphur and FAD centres in protein C.



3.1.E. Interaction of protein C with a platinum redox electrode

Protein C interactions with a redox electrode were investigated, with a view to examining the ability of protein C to equilibrate with a redox electrode in the absence of mediator dyes, small electron carriers used to catalyze electron transfer between redox electrodes and redox centres of proteins (e.g. Wilson *et al.*, 1970; Ohnishi, 1973). Protein C might be interesting in this respect because it has NAD(P)H-acceptor reductase activities which are independent of the other methane monooxygenase components (Colby and Dalton, 1979), and the ability to catalyze electron transfer from NAD(P)H to a redox electrode (or a redox dye attached to a transparent membrane, for review see Mazur, 1983) could be useful as part of a biosensor involving NAD(P)H (for review see Scantland and Gatz, 1983), for example. Another interesting biotechnological possibility is the use of protein C for the production of NAD(P)H from NAD(P)⁺, which is examined in 4.2.D., since there are few systems capable of performing this reaction at a reasonable rate (e.g. Payen *et al.*, 1983). One of the problems is that of uncoupling unwanted acceptor-reductase activities, and another is the inability of most proteins with redox centres to interact with a redox electrode, which may be at least partially due to a charged bilayer at the interface of the electrode with the sample solution, called the "Helmholtz double layer" (see Fujihara and Osu, 1977). Since many biochemical reactions use the

cofactors NAD and NADP, a system able to carry out these two reactions, regeneration of reduced cofactors and reduction of a redox electrode, could be of widespread use.

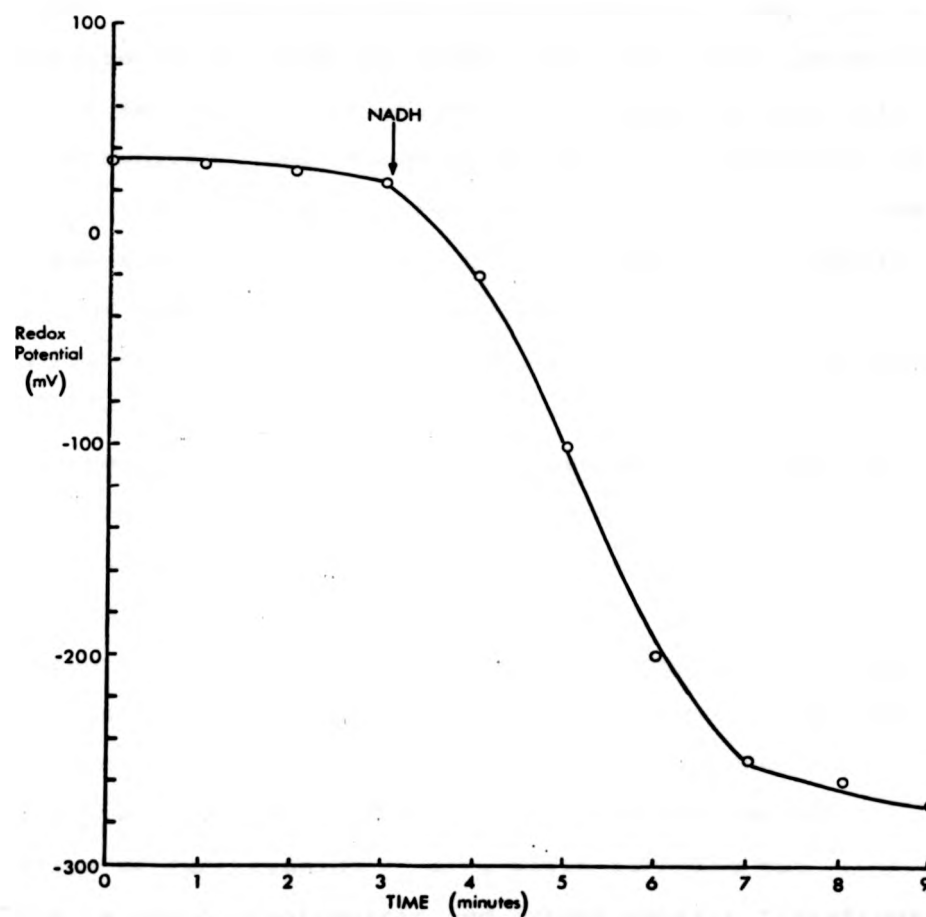
The redox electrode used here measures sample redox potentials relative to a silver / silver chloride reference half-cell, see Materials and Methods. The sample half-cell is comprised of the sample in contact with a platinum electrode, and the two half-cells are in electrical contact via a porous ceramic junction.

(1) Using an anaerobic redox cell (see Materials and Methods), on addition of excess NADH (10 micromoles) to protein C (110 nmoles) under anaerobic conditions, the electrode develops a negative redox potential, equilibrating at around -300 mV within 5 to 10 minutes, see Figure 3.10. Protein C or NADH alone has a negligible effect on the electrode redox potential. The effect is less marked with 30 nmoles of protein C, giving a -120 mV shift in 4 minutes.

With 30 nmoles of apo-iron-sulphur protein C (see 3.2.1.) a -80 mV shift was seen in 3.5 minutes, on addition of excess NADH, so the iron-sulphur redox centre is not necessary for interaction with a redox electrode. The ability of protein C to interact with the redox electrode in this way appears to be quite unusual (see Fujihara and Osa, 1977), since the redox centre in an enzyme may not always approach close enough to the electrode surface for efficient electron transfer to take place.

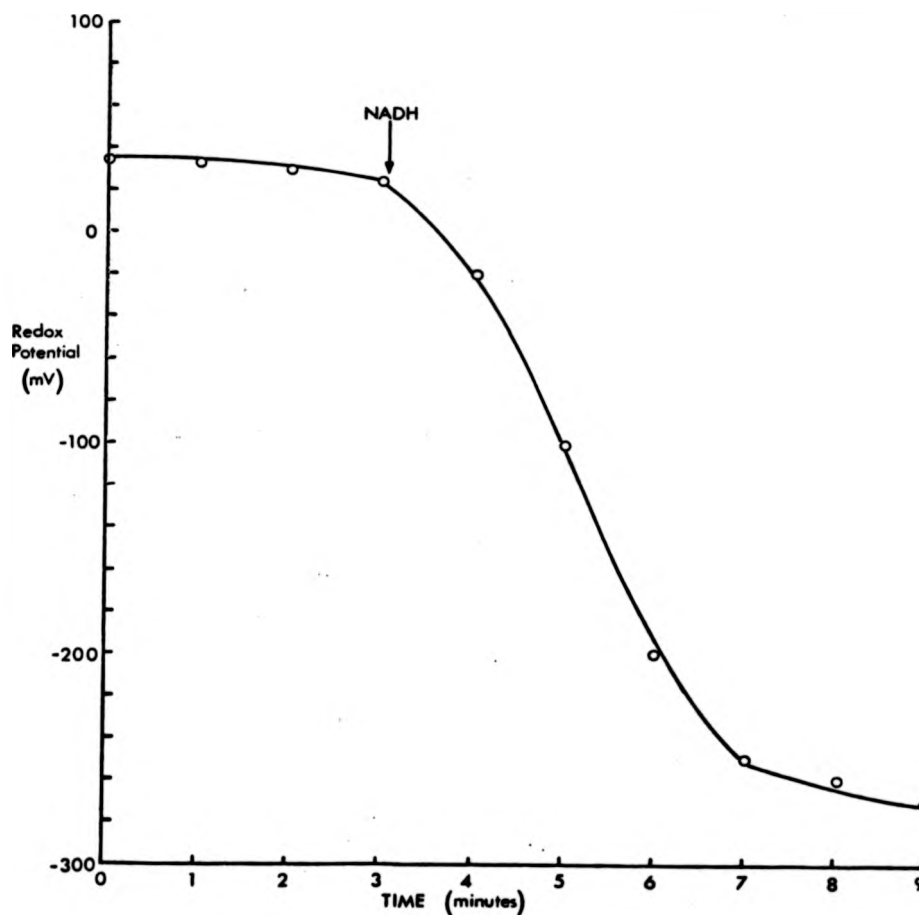
(2) Spectrophotometry was carried out on protein C (240 nmoles in 3 ml of 20 mM Tris-HCl, pH7, 5 mM sodium

Figure 3.10. Reduction of a redox electrode by protein C in the presence of NADH.



To protein C (110 nmoles in 2 ml 20 mM Tris-HCl pH7.0) excess NADH was added (10 micromoles), indicated by the arrow. In the absence of protein C, no shift in redox potential was seen on the addition of NADH.

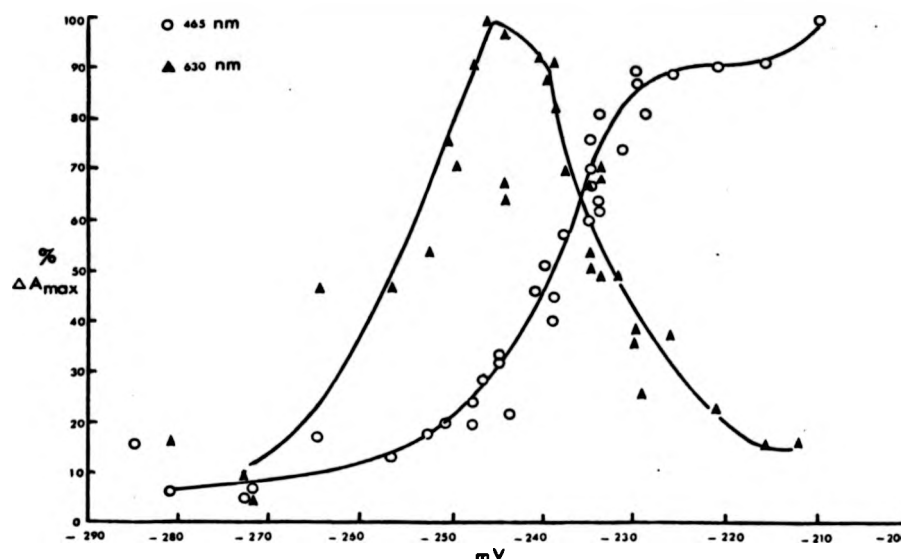
Figure 3.10. Reduction of a redox electrode by protein C in the presence of NADH.



To protein C (110 nmoles in 2 ml 20 mM Tris-HCl pH7.0) excess NADH was added (10 micromoles), indicated by the arrow. In the absence of protein C, no shift in redox potential was seen on the addition of NADH.

thioglycollate, 4°C) in the anaerobic cuvette system with the redox electrode attached, as detailed in Materials and Methods. The redox potential was shifted with NADH or dithionite to decrease the sample half-cell redox potential, and with ferricyanide or oxygen to increase the half-cell redox potential. Any change in the electrode potential as the titration proceeds may be compared with changes in the absorbance profile of protein C. With sub-stoichiometric levels of NADH, a much decreased and slower shift to negative redox potential was seen, e.g. -70 mV in 8 minutes, as compared with a -300 mV shift seen in the presence of a stoichiometric excess of NADH. It may be that protein C cannot deliver enough current to the redox electrode under these conditions. Protein C remained semi-reduced, as indicated by its absorbance profile, indicating that the efficiency of electron transfer to the redox electrode may be low, since protein C would not fully oxidize under these conditions. Protein C was also reduced with NADH or dithionite in the anaerobic cuvette, and oxidized by addition of ferricyanide or air, while carrying out oxidative spectrophotometric and potentiometric titrations. The range over which protein C can reoxidise is fairly variable, and can be anywhere between -280 mV to -100 mV. The mid-point potentials of the protein C peaks are in reasonable agreement with the estimates found with redox dyes (see 3.1.D.) but the apparent redox range over which reoxidation occurs is often compressed, with most of the oxidation in the -200 to -230 mV region (see Figure 3.11.),

Figure 3.11. The potentiometric oxidative titration of protein C.



Protein C (260 nmoles in 3.5 ml 20 mM Tris-HCl pH7) was reduced with a stoichiometric excess of dithionite (100 microlitres of 2 mM dithionite in 0.25 M Tris-HCl pH7.6) in the anaerobic cuvette, and reoxidised by the addition of aliquots of air. Oxidation was monitored in the SP8-200 spectrophotometer, and using a redox electrode. Very similar results were seen with excess NADH as an initial reducing agent instead of dithionite.

although the range can also be around 120 mV. Perhaps this narrow range is caused by lack of equilibration of protein C with the redox electrode, or poisoning of the electrode due to the presence of oxygen (R.Cammack, personal communication). No difference was seen with either NADH or dithionite as the initial reducing agent. These data appear to indicate a partial interaction of protein C with a redox electrode, since there are no obvious mediators present.

In summary, it appears that protein C is capable of transferring electrons to a redox electrode without a requirement for mediators, although it does not seem to be able to equilibrate fully with the redox electrode used here.

3.2. The functions of the redox centres in protein C

3.2. Introduction

One method used to investigate the roles of redox centres in proteins has been to remove specifically or inactivate a particular redox centre, and to attempt to correlate this lack of a (functional) redox centre with biological activities. The correlation may be taken further by attempting to replace or reactivate the redox centre in question, and demonstrating the return of any biological activities which were previously lacking. This technique, known as reconstitution, can be more difficult for proteins with multiple redox centres, such as protein C, because any redox centre inactivation must then be specific to a particular redox centre (for a review of iron-sulphur reconstitutions in iron-sulphur flavoproteins see Singer et al. 1973; for selective flavin replacement in a two redox

centre enzyme. NADPH-cytochrome P-450 reductase, see Vermilion and Coon. 1978; Vermilion *et al.*, 1981).

Selective reconstitutions have been attempted on protein C, namely specific removal and regeneration of the iron-sulphur centre of protein C, and removal and replacement of the FAD redox centre from the resulting apo-iron-sulphur protein C, both by simple and established methods. Correlations between the presence of a functional redox centre and enzymic activities may be interpreted to give information on the roles of the two redox centres in protein C.

3.2.1. Destruction and reconstitution of the iron-sulphur centre of protein C

The iron-sulphur centre of protein C can be destroyed using the mercurial mersalyl (see 2.3.B(2c).), as was first used by Malkin and Rabinowitz on Clostridial ferredoxin in 1966 (for review see Malkin, 1973; also see 2.3.B(1b).), following the findings of Lovenberg *et al.*, (1963), that mercurials could bleach the ferredoxin, releasing iron and sulphide. Mersalyl reacts covalently both with the cysteines and the sulphides of the iron-sulphur centre; on a stoichiometric basis 1 mole of monovalent mercurial reacts with 1 mole of cysteine and 2 moles of mercurial per mole of sulphide, a total of 8 moles of mercurial per Fe_2S_2 centre (Petering and Palmer, 1970; for review see Malkin, 1973). However, protein C required excess mersalyl to effect destruction of the iron-sulphur centre, see Figure 3.12. The absorbance changes during the

centre enzyme, NADPH-cytochrome P-450 reductase, see Vermilion and Coon, 1978; Vermilion *et al.*, 1981).

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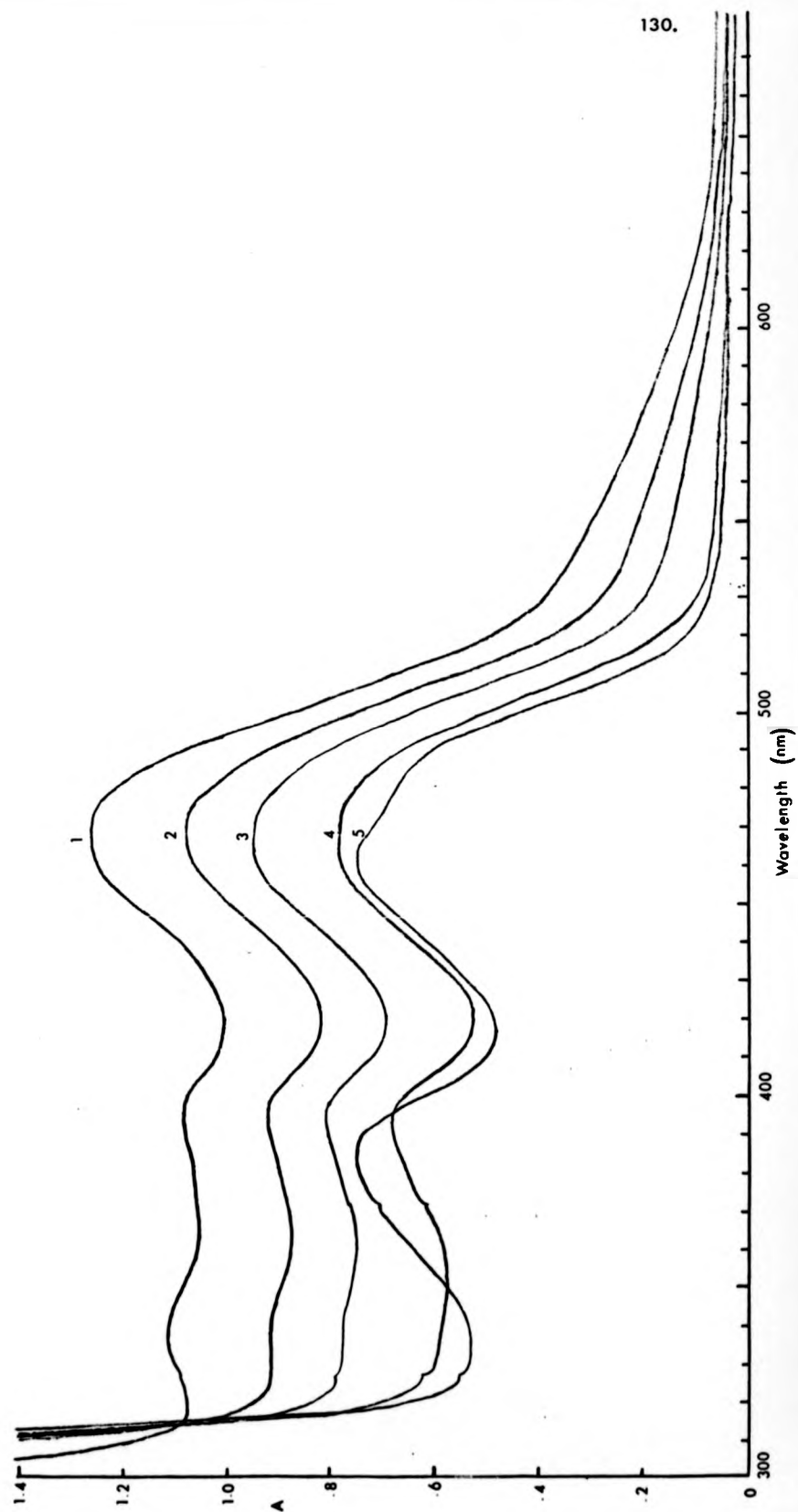
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titration were rapid, and the absorbance was stable between the additions of mersalyl. During the titration, both MMO and the NADH-acceptor reductase activities declined in parallel with the loss of absorbance due to the destruction of the iron-sulphur centre, so titration with mersalyl fails to resolve the NADH-acceptor reductase and MMO activities of protein C. It may be that modification of one or more cysteine residues of the iron-sulphur centre destroys NADH-acceptor reductase activities, because mersalyl destroys this activity of protein C when no iron-sulphur centre is present. This result is in contrast to that seen with the iron-sulphur flavoprotein dihydroorotate dehydrogenase, where treatment with small amounts of mersalyl destroyed the iron-sulphur centre (as indicated by EPR) and the dihydroorotate dehydrogenase activity, but not the NADH-oxidase activity (Aleman *et al.*, 1968). Towards the end of the titration, the FAD absorbance appears to undergo a shift and absorbance increase in the 350 nm to 390 nm region, indicating that mersalyl is affecting the environment of the FAD group. The resultant apo-iron-sulphur protein C lacked both MMO activity and

Figure 3.12. The titration of protein C with mersalyl.

Protein C (96 nmoles in 0.7 ml 20 mM Tris-HCl pH7.0) was titrated with 4, 6, 8, and 18 microlitres of 69 mM mersalyl (in 0.25 M Tris-HCl pH7.6), in total a 12.6 fold excess of mersalyl over the protein C concentration, though most of the changes seen had occurred with a 5.6 fold excess of mersalyl over protein C.



NADH-acceptor reductase activities, and was reduced only very slowly on spectrophotometric titration with NADH, and on anaerobic reduction with dithionite the semiquinone absorbance appeared to be depressed, further indicating that excess mersalyl causes alterations to the environment of the FAD group.

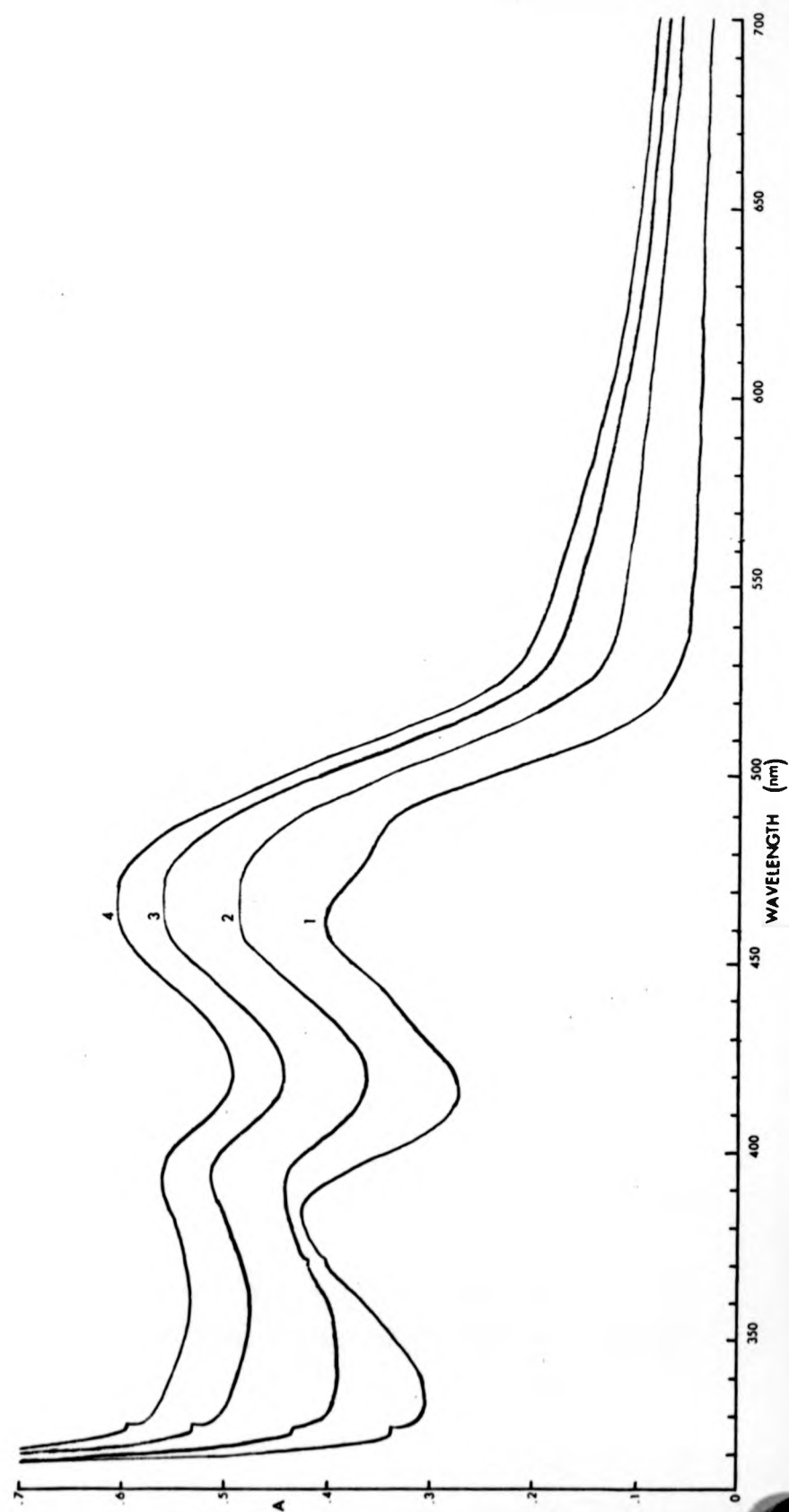
Lovenberg et al., (1963) found that treatment of the bleached ferredoxin with 2-mercaptoethanol without prior separation from the mercurial restored the ferredoxin colour, although it did not result in a complete reconstitution of the ferredoxin (for a review of the reconstitution of this and other ferredoxins, see Malkin, 1973). Treatment of protein C with excess 2-mercaptoethanol (250 to 300 nmoles), followed by incubation for 30-40 minutes after which no further absorbance increases occur, appears to reconstitute the iron-sulphur centre, and results in restoration of both the MMO and the NADH-acceptor reductase properties (Table 3.2.), although the iron-sulphur centre absorbance does not seem to be fully restored (around 80% in Figure 3.13., and 88% over 40 minutes in a similar experiment), in agreement with Lovenberg et al., (1963), see Figure 3.13., presumably indicating incomplete iron-sulphur complex formation. Excess mersalyl and 2-mercaptoethanol, a potent inhibitor of the MMO, were then removed by ultrafiltration over a PM30 membrane. Complete removal of 2-mercaptoethanol is required because it gives a high endogenous NADH-acceptor reductase activity with NADH and DCPIP. Therefore the wash from the PM30 was used as a

control, to ensure that all 2-mercaptoethanol had been removed from the protein C sample.

However, if the mersalyl treated protein C is desalted, either by ultrafiltration and washing with 20 mM Tris-HCl pH7, over a PM30 membrane (30,000 molecular weight cut-off) or on a Sephadex G-15 column, on addition of excess 2-mercaptoethanol no iron-sulphur reconstitution takes place, as indicated spectrophotometrically, probably due to the lack of one or both of the iron-sulphur constituents. Malkin and Rabinowitz (1966) reported the removal of mersalyl-sulphide from Clostridial ferredoxin by desalting on Sephadex G-25, so the same situation may hold for apo-iron-sulphur protein C. The resulting apo-iron-sulphur protein C, which has had the covalently bound mersalyl displaced by excess 2-mercaptoethanol, was then desalted by ultrafiltration. Once separated from the 2-mercaptoethanol and the mersalyl, the apo-iron-sulphur protein C showed a very low level of MMD activity (on a specific activity basis), but retained much of the NADH-acceptor reductase activities (see Table 3.2.), and appears to be very similar to protein C in its interaction with NADH, both by equilibrium redox investigations, and by steady-state and

Figure 3.13. Reconstitution of mersalyl-treated protein C by 2-mercaptoethanol.

Protein C was titrated with mersalyl as for Figure 3.12. 0.7 ml of this protein C (57 nmoles) was treated with 20 microlitres of 2-mercaptoethanol. No further absorbance changes occurred after 30 minutes, (4).

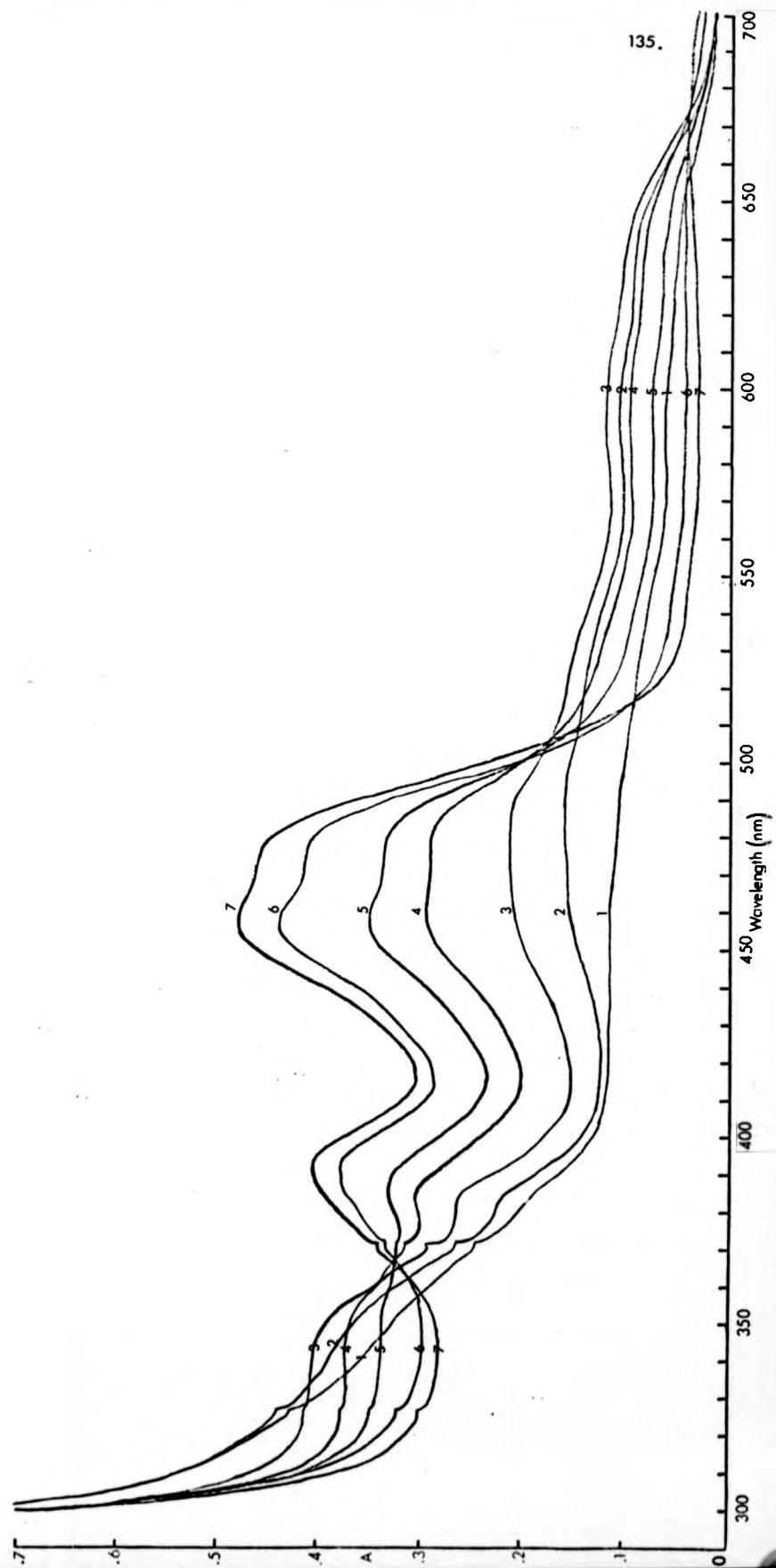


transient kinetics (see 4.1.C. and 4.2.A.). One observation of interest is the occasional large increase in MMO specific activity seen on reconstitution of the iron-sulphur centre, giving specific activities 2 to 3-fold greater than the native protein C. It may be that some part of the reconstitution process acts to restore inactivated groups on protein C, e.g. oxidation of the sulphur groups of protein C might be reversed by the reconstitution process. One such preparation had only half the native iron-sulphur absorbance, after desalting to remove mersalyl and mecaptoethanol, indicating that the amount of iron-sulphur groups (on an absorbance basis) need not necessarily limit protein C MMO activity.

Redox titration of apo-iron-sulphur protein C (see Figure 3.14.) indicates that the semiquinone form of the FAD is still present but there appear to be some changes as compared to the behaviour of the FAD redox centre on reduction of native protein C. At the stage of reduction where the level of semiquinone is maximal, there is appreciable reducible absorbance at 465 nm, in contrast with native protein C where the reducible absorbance at 465 nm is due to the iron-sulphur centre. Additionally, there is a much larger semiquinone contribution in the 340 nm region

Figure 3.14. Oxidative titration of apo-iron-sulphur protein C.

Apo-FeS protein C (0.1 mM) was made anaerobic in a semi-micro cuvette with a Suba Seal, and then reduced with excess buffered dithionite,(1) Spectra were recorded on reoxidation with air,(2-7).



than occurs with native protein C (about double that seen with native protein C on an FAD concentration basis), whereas the semiquinone absorbance contributions at 580 nm and 630 nm are about the same as for native protein C on an FAD concentration basis. These two changes are consistent with a significant proportion of the iron-sulphur centres in native protein C being mainly on the same protein C molecules as the FAD redox centres, a result which is also consistent with the enhanced relaxation of the semiquinone by the reduced iron-sulphur centre, seen by EPR (see 3.1.B; 3.1.C.). The residual absorbance at 465 nm may indicate that the iron-sulphur centre promotes quantitative conversion of the quinone to the semiquinone form in native protein C.

Table 3.2. Reconstitution of the iron-sulphur centre of protein C.

| | MMD (%)* | reductase (%)* |
|--|----------|----------------|
| native protein C | 100 | 100 |
| apo-Fe ₂ S ₂ protein C | 3 | 85 |
| reconstituted protein C | 90 | 95 |

* Activities are expressed relative to native protein C on a specific activity basis, 6 micromoles ethylene oxide minute⁻¹ mg⁻¹ protein C for MMD assays and 50 micromoles DCPIPH₂ minute⁻¹ mg⁻¹ protein C for the NADH-acceptor reductase assay. Parallel activities were found with ferricyanide and cytochrome c as acceptors.

Therefore NADH-acceptor reductase activities do not require the iron-sulphur redox centre, indicating that the FAD redox centre in protein C interacts with NAD(P)H directly and does not require the iron-sulphur centre to oxidize NAD(P)H since the presence or absence of this centre has no effect on NADH-acceptor reductase activity. However, the presence of the iron-sulphur centre is required for MMO activity, and this suggests that the iron-sulphur redox centre may be necessary for electron transfer from protein C to protein A, and further evidence supporting this view is reported in 4.3.B. It is interesting to note that treatment of apo-iron-sulphur protein C with mersalyl destroys NADH-acceptor reductase activities (as occurs with native protein C) and modifies the spectrum of this form of protein C to that seen on treatment of native protein C with mersalyl, indicating that the modification of one or more cysteine thiol groups blocks initial electron transfer from NADH to the FAD group of protein C, and explaining why mersalyl-treated protein C is reduced only very slowly by NADH during anaerobic titrations. With dithionite as reducing agent, the semiquinone absorbance appears to be much depressed, suggesting that mersalyl alters the environment of the FAD group in protein C. This inactivation of apo-iron-sulphur protein C by mersalyl raises the possibility that a cysteine residue might be required for transfer of electrons from NADH to protein C.

3.2.2. Removal and replacement of the FAD redox centre of protein C

The FAD redox centre was removed from native protein C or apo-iron-sulphur protein C (treated with mersalyl, desalted, and treated with 2-mercaptoethanol, resulting in reductase activity, but not MMO activity) by ultrafiltration over a PM30 in the presence of 8 M urea (deionized immediately before use by passage through the mixed-bed ion exchange resin Amberlite MB-1) in 20 mM Tris-HCl pH7 for several hours. Protein C seems to be very stable to high salt concentrations because attempts to remove the FAD group with 2.2 M KBr, pH6.5 (Hiwatashi et al., 1976), had no effect on protein C absorbance or biological activity. On urea treatment of protein C the iron-sulphur centre was destroyed (as happens with Spinach ferredoxin, Petering et al., 1971) as judged by the disappearance of the characteristic yellow-brown colour of protein C, and replaced by a yellow colour identical to that of FAD (which like mersalyl treated protein C, but unlike apo-iron-sulphur protein C after treatment with 2-mercaptoethanol or native protein C, did not fluoresce on exposure to u.v. light). Removal of the FAD group from protein C led to a loss of NADH-acceptor reductase activity which was restored on addition of a 5-fold excess (500 nmoles) of authentic FAD in 20 mM Tris-HCl, pH7, followed by incubation for 40-60 minutes at 4°C, giving a 5 to 12-fold stimulation of NADH-acceptor reductase activity, see Table 3.3. The specific activity was variable relative to native protein C, giving 40% to 180% of the NADH-acceptor reductase

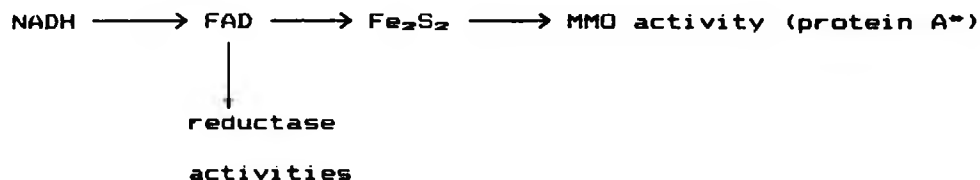
Table 3.3. Reconstitution of the FAD group of protein C.

| | MMO (%)* | reductase (%)* |
|---|----------|----------------|
| native protein C | 100 | 100 |
| apo-Fe ₂ S ₂ protein C | 3 | 85 |
| apo-Fe ₂ S ₂ -apo-FAD protein C | 0 | 11 |
| apo-Fe ₂ S ₂ protein C (reconstituted) | 2 | 110 |

* Activities are expressed relative to native protein C on a specific activity basis, 6 micromoles ethylene oxide minute⁻¹ mg⁻¹ protein C for MMO assays and 50 micromoles DCIPH₂ minute⁻¹ mg⁻¹ protein C.

activity (DCPIP) of native protein C. However, the MMO activity of this apo-iron-sulphur protein C is still very low (0-3%). Therefore there appears to be a correlation between NADH-acceptor reductase activity and the presence of the FAD redox centre, indicating that the FAD redox centre is necessary for this enzymic activity.

In summary, there appears to be a correlation between the presence of the FAD and iron-sulphur redox centres and the NADH-acceptor reductase and MMO activities respectively, suggesting the following order for electron flow (further evidence for this order is examined in 4.3.B.):-



* See 4.3.B. for further evidence of this order.

The order is consistent with protein C being a 2-electron / 1-electron transformase (Hemmerich, 1977), where the second redox group, the iron-sulphur centre, acts as a 1-electron carrier to provide single, constant potential electrons to the acceptor. Either FMN or iron-sulphur centres play similar roles in other systems, as detailed in section 1.3.A.

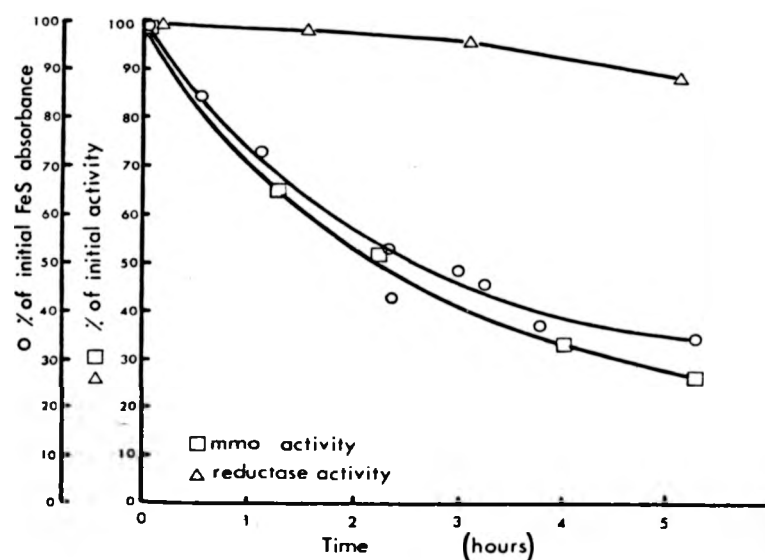
3.2.3. The relationship between iron-sulphur centre stability and biological activities of protein C

Another method was attempted in order to study the biochemical roles of the two redox centres, by examining the relationship between iron-sulphur centre stability as monitored by absorbance (at 465 nm) and the stability of protein C enzymic activities. The FAD optical contribution appears to be very stable, unlike the iron-sulphur contribution, determined using mersalyl to destroy this redox centre, so absorbance decreases may be assigned to decay of the iron-sulphur centre. A less stable protein C (see 2.3.C.) was initially used for these experiments, which decayed at about 5% per hour at 4°C in the presence of 5 mM

sodium thioglycollate. The decay of the iron-sulphur centre monitored optically shows a correlation with the decay in MMO activity, unlike the NADH-acceptor reductase activity which remained fairly stable, see Figure 3.15. This suggests that NADH-acceptor reductase activity is independent of the iron-sulphur centre integrity and that this enzymic activity need not coparallel MMO activity for protein C. This observation is consistent with the finding of Colby and Dalton (1979) that NADH-acceptor reductase activities may be retained when MMO activities have been lost, and is consistent with the FAD group alone being required for NADH-acceptor reductase activities, but that the iron-sulphur centre is required for MMO activity (electron transfer to protein A, see 4.3.B.). It is of interest that the present author has never seen a protein C preparation lacking MMO activity, and this may be due to the earlier inclusion of sodium thioglycollate in the purification scheme for protein C (see 2.2.A. and 2.3.C.). The optical and MMO stability of this more unstable protein C fell off as the sodium thioglycollate concentration was lowered, giving initial rates of decay in MMO activity of 14%, 50%, and 100% hour⁻¹ in the presence of 5 mM, 3 mM, and 0 mM sodium thioglycollate respectively, with similar decreases in the iron-sulphur absorbance. Therefore it appears that thioglycollate stabilizes this protein C MMO activity by its action on the iron-sulphur centre, perhaps by slowing down its decay or by mediating its reconstitution. This last possibility is evidenced if mersalyl-treated protein C is desalted by ultrafiltration

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Figure 3.15. Resolution of MMO and reductase activities in an unstable protein C.



The protein C sample was at a temperature of 17°C and a concentration of 3 mg/ml. MMO activity was assayed with ethene as substrate, and the initial specific activity was typical, at around 6 units. Reductase activities were assayed using DCPIP (40 units) alone, though previous reductase assays with this preparation had never resolved the DCPIP, ferricyanide, or cytochrome c activities.

with a PM30 membrane, in the presence of 5 mM sodium thioglycollate and 20 mM Tris-HCl pH7, when iron-sulphur absorbance contributions return and appreciable MMO activity is found.

However, stability studies with a more stable protein C (decaying in activity at around 1% per hour) do not appear to resolve the MMO and NADH-acceptor reductase activity, since both activities decay in parallel, but at a reduced rate as compared with the more unstable protein C, and moreover there is no longer any significant fall-off in the absorbance of the iron-sulphur centre, indicating that factors other than iron-sulphur centre stability are responsible for the decay of enzymic activities. The NADH-acceptor reductase activity of apo-iron-sulphur protein C decays at a similar rate to native protein C, corroborating this view, and was also stabilized by thioglycollate to the same extent as native protein C. Thioglycollate might act both to repair the iron-sulphur centre and to reactivate oxidized cysteine residues required for NADH acceptor reductase activities.

In summary, more unstable protein C preparations may be limited with respect to MMO activity by the stability of the iron-sulphur redox centre, but this does not appear to be the case for more stable protein C preparations.

CHAPTER 4. Interaction of protein C with NADH and protein A

Introduction

Electron transfer from NADH to protein C and from protein C to protein A may be investigated both by equilibrium and kinetic methods. Equilibrium techniques used here include anaerobic spectrophotometric titrations of reduced protein C with protein A, to see if electron transfer takes place on an equilibrium basis. A demonstration of electron transfer by this method is simply consistent with electron transfer between the proteins with respect to the enzyme mechanism, and to prove that any electron transfer is part of the MMO mechanism requires quantitation of the rate of electron transfer. The rate of electron transfer must be shown to be at least as fast as the slowest step (the rate-limiting step, which dictates the overall MMO rate) of the overall reaction. There are several readily available and simple kinetic methods which may be used to give information about the intermediates on the electron transfer pathway and their rates of interconversion, namely steady-state and transient kinetics, and results from these two techniques may be combined to build up a picture of the reaction mechanism. Simple steady-state kinetics, with catalytic amounts of protein C, can be used to show consistency with the presence of intermediates and to determine the maximum rates of interconversion of intermediates. Steady-state kinetics may also be used to look at reactions of protein C which are independent of the MMO activities, namely the NADH-acceptor reductase activities, to give information on steps preceding electron transfer to protein A, to indicate the extent to

which Protein C reduction by NADH is rate-limiting to the MMO reaction as a whole, and also to indicate any possible steps that might be rate-limiting to the reduction of protein C by NADH.

Electron transfers involving the two redox centres of protein C may be examined directly with stoichiometric amounts of protein C. Rapid electron transfer events can be followed on a millisecond time scale using the stopped-flow technique, on an apparatus similar to that designed by Gibson and Milnes (1964). In this technique, reactants are mixed rapidly by passage through a mixing chamber, and are then monitored for transmittance in an adjacent sample cavity, approximately 2-5 milliseconds after the reactants have been mixed, using a rapid response photomultiplier. In the apparatus used here, the storage oscilloscope is triggered when the plunger of the stopping syringe, adjacent to the sample chamber, activates a microswitch at the end of the plunger's path, and can then monitor solution ageing. Protein C is amenable to investigation by this technique because its redox centres undergo absorbance changes on reduction or reoxidation. The technique allows direct identification of transient intermediates and rate constants for the interconversion of intermediates. Partial reactions of protein C, such as reduction of the redox centres by NADH, may be monitored and quantitated by this technique. A combination of the three techniques can be used to build up a simple kinetic description of the interaction of protein C with its biological electron donor and acceptor, including the pathway taken by electrons from NADH to protein A.

4.1.A. The interaction of protein C with NADH

4.1.A(1). Putative intermediates of reduced protein C

Colby and Dalton (1979) reported that protein C could be reduced directly by NADH in an equilibrium spectrophotometric titration. This, together with the NAD(P)H-acceptor reductase properties of pure protein C (Colby and Dalton, 1979), was taken to indicate that protein C was the component of the MMO responsible for interaction with NAD(P)H. Stopped-flow has been used to investigate this interaction directly, reacting NADH with protein C and monitoring the rate and extent of reduction at several wavelengths. The author was instructed in the use of the stopped-flow apparatus by Dr.P.Moore, the designer of this particular apparatus.

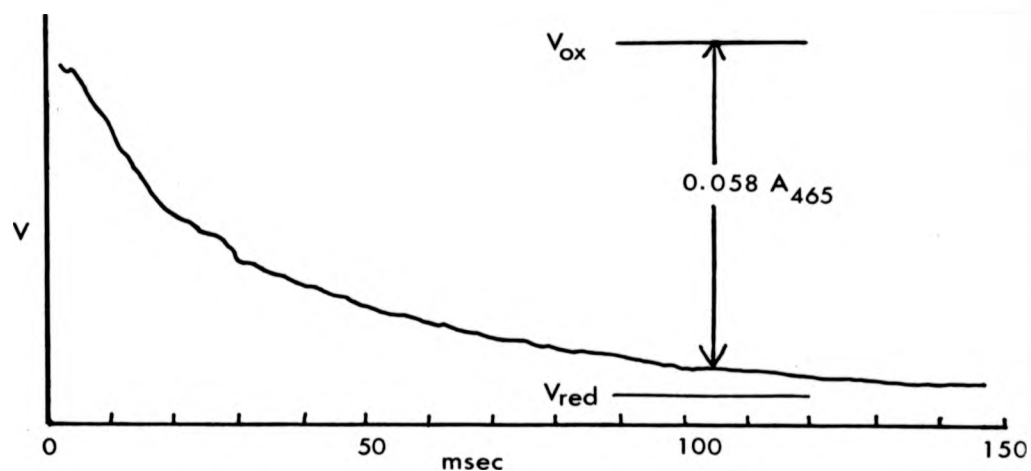
(a) Protein C was reacted (0.4 ml with a final concentration of 11.25 micromolar) with approximately stoichiometric levels of NADH (0.4 ml with a final concentration of 5.65 micromolar), in 20 mM Tris-HCl pH7, at room temperature. Reduction in this single turnover experiment was monitored at 465 nm, setting the wavelength by means of a monochromator adjacent to the light source. This wavelength was chosen because both the quinone and the iron-sulphur centre make significant reducible absorbance contributions, the quinone form of FAD about 2/3 of the total absorbance change at 465 nm and the iron-sulphur centre about 1/3 of the total absorbance change (see Figure 3.3.), in addition to which the 465 nm peak has the largest reducible

absorbance change of the protein C absorbances. The experiment was done under aerobic conditions, because NADH-oxidase activities (0.09 units; Colby and Dalton, 1979) of protein C are very much less than activities with other electron acceptors such as DCPIP (50 units), indicating that protein C may be reduced much more rapidly by NADH than it is oxidized by O_2 .

The resultant photomultiplier voltage decrease was recorded on a storage oscilloscope (typically with a full scale voltage of 0.5 V and over a time scale of 0.5 seconds), and also on a recorder of transients (Datalab), so that accurate copies of traces were available for quantitation. The voltage recorded by the photomultiplier is directly proportional to the transmittance of the sample solution, and if the voltage given by 0% transmittance is found (when the light beam is obscured) relative to that of the sample solution, the change in transmittance with time may be found, and first order rate constants can be calculated by plotting $\log_{10}[\log_{10} V_{\infty}/V_t] \rightarrow \text{time } (t)$, when a first order process gives a linear slope equal to $-k / 2.303$. If absorbance values are required, the absolute transmittance may be found by measuring the voltage in the presence of buffer (100% transmittance), relative to the voltage given at 0% transmittance.

On reaction with NADH, protein C was reduced rapidly at 465 nm, with a first-order rate constant of $25 \pm 6 \text{ sec}^{-1}$. The reaction appeared to be over largely within 100 milliseconds, see Figure 4.1., and underwent little

Figure 4.1. The reduction of protein C with NADH by stopped-flow.



Protein C (at a final concentration of 11.25 micromolar, in 20 mM Tris-HCl pH7.0, at room-temperature) was reacted with NADH (at a final concentration of 5.65 micromolar). The reduction was monitored at 465 nm, at 0.5 seconds full scale and 0.5 V full scale. V_{ox} corresponds to the fully oxidised protein, which underwent no further absorbance change, and V_{red} corresponds to the minimum absorbance seen.

further reduction on longer time scales. Individual traces were fitted well by a first-order rate constant to within a second (by the method of least squares), although there was considerable variation between the rate constants at this concentration of NADH. At around $20\text{--}30\text{ sec}^{-1}$, this rate constant is considerably less than the steady-state rate constant, k_{cat} , for several of the NADH-acceptor reductase assays (seen with saturating NADH), even when these are corrected for the number of reducing equivalents involved, and about equal to the rate constant seen with DCPIP (see 4.2.). E.g. at 250 units, the ferricyanide assay corresponds to a k_{cat} of 154 sec^{-1} (the rate constant for turnover of ferricyanide on the enzyme), which is a one electron reduction process, so this rate constant should be halved for comparison with the two electron reduction process seen on reduction of protein C at 465 nm and indicates that protein C may not be reduced at a maximal rate at this concentration of NADH (see 4.1.A(2).).

Protein C was observed to reoxidize on a tens-of-seconds time scale at this concentration of NADH, so that reoxidation, monitored by the oscilloscope (and also by the photomultiplier anode current) was complete within a few minutes. With twice the previous dose of NADH, protein C was reduced slightly more rapidly, but qualitatively gave the same size overall absorbance change both on reduction and reoxidation (which takes considerably longer than with half the amount of NADH). It is of interest that the fully oxidized protein C absorbance (voltage) was often less

than the voltage at "time zero", indicating that appreciable reduction of protein C had taken place within the dead-time of the apparatus. Therefore, a qualitative conclusion is that maximal reduction of protein C at 465 nm has occurred in a short space of time, and no further slow absorbance decrease was seen on a time-scale similar to that attained during equilibrium experiments. This conclusion is important because both redox centres of protein C have a significant reducible absorbance at 465 nm, and this result indicates that both centres may be fully reduced within a short space of time, much more quickly than the protein C maximum specific activity in the MMO reaction (around 3.7 sec^{-1} , calculated from a specific activity for protein C of 6 units, for epoxyethane production). The absorbance of the fully oxidized and fully reduced protein C forms was quantitated (0.1124 A and 0.0541 A respectively), and compared with the absorbance change seen at 465 nm on full reduction of the same concentration of protein C in the spectrophotometer (a 0.064 A decrease from 0.116 A). The two sets of absorbances were in good agreement (the path length of the observation chamber in this stopped-flow apparatus is 1 cm, and indicated that the rapid absorbance decrease seen on reduction by stopped-flow was 90% of the total equilibrium absorbance change seen on reduction. However, both these absorbance changes are less than would be calculated from the Beer-Lambert law (67% and 74%) for this concentration of protein C, based on extinction coefficients derived using a circa 10-fold greater

concentration of protein C (3.1.B.). Nevertheless, since the observed kinetic absorbance change was considerably greater than $2/3$ of the equilibrium absorbance change seen on reduction, and any changes seen occurred rapidly (with enough NADH to produce the 2-electron form of protein C, see 3.1.B.2.), it seems very likely that both the iron-sulphur centre and the quinone are strongly reduced in a short space of time, at this concentration of NADH. For example, if the quinone was not reduced rapidly, only $1/3$ of the equilibrium absorbance change (due to the iron-sulphur centre) would be seen by stopped-flow.

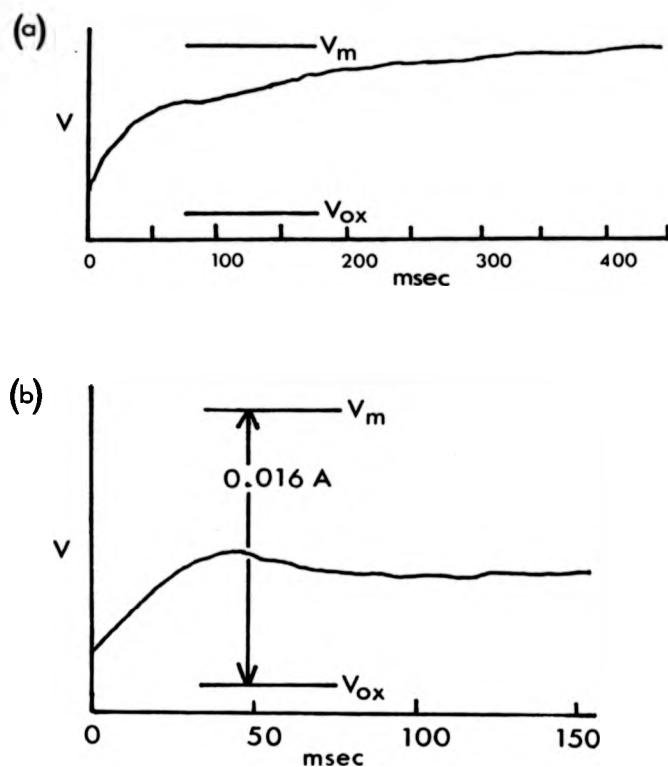
In summary, the kinetic absorbance change seen on reduction of protein C indicates that both the iron-sulphur and the FAD redox groups are reduced much more rapidly than the rate-limiting step for protein C in the MMO reaction (even at this NADH concentration). The definition of a prosthetic group is that of a tightly bound coenzyme which participates in the catalytic cycle of an enzyme. One of the criteria used to judge whether an intermediate is on the reaction pathway is whether it is formed faster than or equal to the rate-limiting step of the reaction. The evidence here is consistent with both the quinone and the iron-sulphur centre being prosthetic groups and their reduced forms being intermediates on the MMO pathway. The second criterion is that the putative intermediate should be removed faster than or equal to the rate-limiting step for the reaction (see 4.3.B.). Finally, reduction of protein C by this concentration of NADH will not be rate-limiting to

the MMO reaction if protein C behaves in the presence of the other MMO components as it does here (see 4.3.B.).

(b) Preliminary observation of protein C reduction at 580 nm or 630 nm, where the semiquinone absorbance makes major contributions. was carried out at 0.2 V full-scale deflection and a time scale of 0.5 seconds. With sub-stoichiometric amounts of NADH (5.65 micromolar and 11.25 micromolar protein C), the semiquinone form appears with a first-order rate constant similar to that seen for reduction at 465 nm, greater than the rate constant (3.7 second^{-1}) calculated from the maximum specific activity of protein C in the MMO assay, at 6 units. There is a definite increase in absorbance, qualitatively the trace travels upwards, whereas at 465 nm the trace falls off on reduction.

On oxidation of protein C, the semiquinone absorbance slowly decreases to a constant value (V_{ox}), Figure 4.2(a). With a higher concentration of NADH (21 micromolar final concentration), a decreased amount of semiquinone is formed (see Figure 4.2(b)), with a first-order rate constant similar to that seen for the 465 nm absorbance decrease, at 50 to 70 sec^{-1} . However, after some minutes the absorbance increases to a maximum, and then after some time, falls back to the fully oxidized level. This behaviour would be expected if protein C was rapidly and strongly reduced by excess NADH, past the maximal semiquinone stage, and on oxidation of the dihydroquinone form to the semiquinone form, the maximal semiquinone level is attained. At this

Figure 4.2. Semiquinone formation by stopped-flow.



(a) Protein C (at a final concentration of 11.25 micromolar) was reacted with NADH (5.65 micromolar), monitoring reduction at 630 nm. The absorbance increased to a maximum at V_m , and fell off to V_{ox} on oxidation. The absorbance change was not determined, but might be expected to be around 0.01 to 0.015 A. Recording conditions were 0.5 sec full scale and 0.2 V full scale.

(b) Protein C (11.25 micromolar) was reacted with 21 micromolar NADH, monitoring reduction at 580 nm. The absorbance increased to V_m on oxidation, and then fell off to V_{ox} . Recording conditions were 0.2 sec full scale and 0.2 V full scale.

stage the semiquinone appears to be semi-stable to oxidation, probably because the iron-sulphur centre is oxidised in preference to the semiquinone, and only after this stage does the semiquinone absorbance decrease.

Therefore it appears that protein C may be quickly and rapidly reduced to the dihydroquinone stage without a full intermediate semiquinone stage:- One explanation of this behaviour may be that the rate constant for electron transfer from the FAD to the iron-sulphur centre is similar to the maximum rate constant for reduction of the FAD by NADH (at around 170 sec^{-1}). Then, by the time the dihydroquinone has transferred one electron to the iron-sulphur centre (see 3.2.), further reducing equivalents are available to the FAD, from NADH. Electron transfer between protein C molecules is also a possibility. Protein C may be able to interact rapidly with more than one NADH, since full reduction of protein C requires 3 electrons and NADH is a 2 electron donor, and also protein C molecules may be interacting with each other, in order to account for the odd-electron forms of protein C. If protein C has 1 site for NADH interaction per protein C molecule, then the first NAD⁺ molecule would have to vacate this site for the second NADH molecule to complete reduction of protein C. This rapid replacement would require loose binding of NAD⁺ to reduced protein C, which appears to be the case since the K_i for inhibition of the DCPIP NADH-acceptor reductase assay is 5 mM (see 4.2.B.), whereas the K_D for NADH is 10-20

micromolar (see 4.1.A(2).), and also NADH does not cause any appreciable shifts in mid-point potential of the protein C redox centres as compared to reduction with dithionite, or NADPH.

In summary, protein C may be rapidly and strongly reduced past the 2-electron form by NADH, for which the simplest explanation is that NAD^+ exits rapidly from reduced protein C, allowing a second NADH to bind. The semiquinone form can be formed as quickly as the quinone and the iron-sulphur centre are reduced, and therefore satisfies a criterion for being an intermediate on the electron transport pathway from NADH to protein A, in that it is formed faster than the maximal rate for protein C MMO activity. However, with a stoichiometric excess of NADH, the semiquinone form may be bypassed and protein C may proceed through to the dihydroquinone.

4.1.A(2). The dependence of the reduction rate for protein C on the NADH concentration

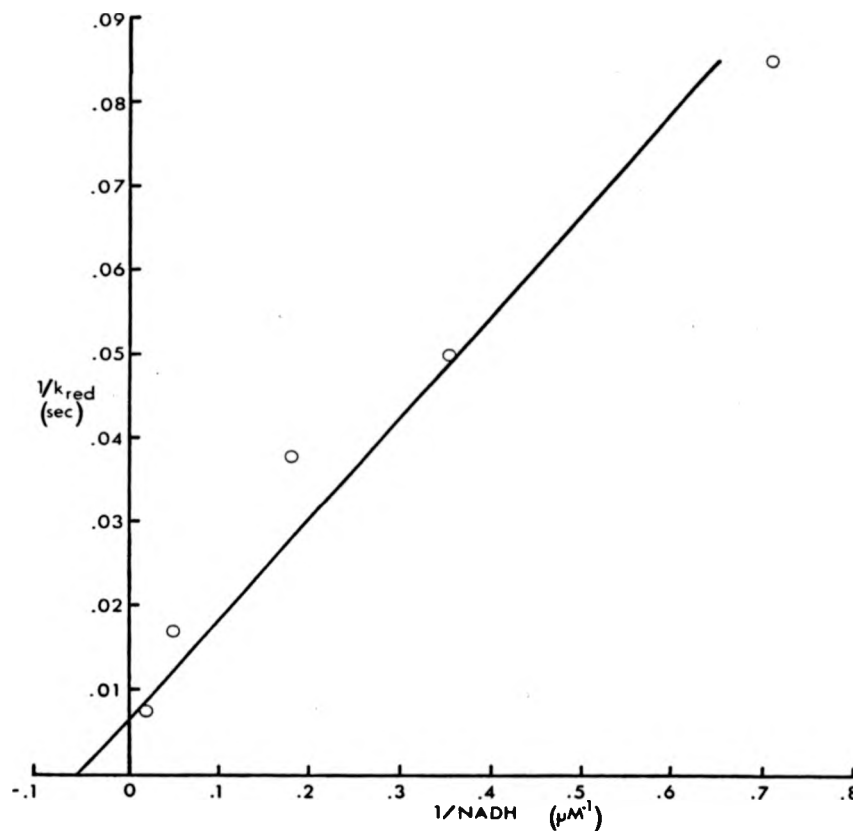
Protein C (a final concentration of 9.4 micromolar) was reacted with different concentrations of NADH (a final concentration of 1.4 to 56.5 micromolar), monitoring reduction at 465 nm. As the concentration of NADH was increased, the reduction appeared to be complete within shorter times, and more reduction occurred within the dead-time of the apparatus. Oxidation of the reduced protein C also took correspondingly longer with increased amounts of NADH (up to about 6 minutes before oxidation was

observed). A plot of $1 / k_{\text{reduced}} \rightarrow 1 / \text{NADH}$ gave a linear slope (k_{reduced} is the first-order rate constant for reduction), see Figure 4.3. This type of hyperbolic dependence of the rate constant on the NADH concentration is consistent with a mechanism in which there is a rapid equilibrium of protein C with NADH followed by a slow reduction of protein C by NADH (for review see Hiromi, 1979):-



The K_d is given by the the negative reciprocal of the x-axis intercept and the first-order rate constant for reduction of protein C is given by the reciprocal of the y-axis intercept, from which the $K_d = 20$ micromolar (see 4.2.B.) and the $k_{\text{red}} = 170 \text{ sec}^{-1}$. The iron-sulphur centre does not appear to be resolved from the FAD centre by this method e.g. the transmittance trace was not biphasic, indicating that rapid electron transfer must be taking place from the FAD to the iron-sulphur centre (see 3.2. and 4.1.C.). The equilibrium constant for electron transfer between these two redox centres is 0.054 for the 1-electron form of protein C (based on the 70 mV difference between the two mid-point potentials of these redox centres, see 3.1.D.), so the reverse rate of electron transfer must around 18.5 times as rapid, since the equilibrium constant is the product of the forward and reverse rate constants. Therefore, since the protein C maximal specific activity is slow on this time

Figure 4.3. Catalytic parameters for reduction of protein C by NADH.



Protein C (at a final concentration of 9.4 micromolar, in 20 mM Tris-HCl pH7.0, and room temperature) was reacted with 1.4 to 56.5 micromolar NADH (final concentration), and the rate of reduction monitored at 465 nm in the stopped-flow apparatus.

scale (and appears to be equal to the rate constant for electron transfer to protein A, see 4.3.B.), the two redox centres may be considered to be in equilibrium. A technique such as rapid-freeze E.P.R., with a lower limit of around 10 msec, would therefore not be expected to resolve the two redox centres, with respect to the order of reduction of the redox centres.

In summary, the maximal rate of reduction of protein C by NADH is around 170 sec^{-1} . This rate constant is significantly faster than the turnover rate constant calculated from the fastest NADH-acceptor reductase activity, found from steady-state kinetics (around 77 sec^{-1} for the ferricyanide assay), indicating that reduction of protein C should not be rate-limiting to its known enzymic activities, in the presence of saturating NADH.

4.1.B. The interaction of protein C with NADD

(4S)-[^2H] NADH was made using materials and a method supplied by Dr. D.E. Griffiths (see Materials and Methods), based on the exchange of the beta-NADH hydrogen atom for deuterium atoms in D_2O , using the transhydrogenase activities of disrupted mitochondria. Stopped-flow experiments were carried out with protein C (at a final concentration of 10 micromolar) as detailed in 4.1.A(2)., varying the NADD concentration (no D_2O was present in the NADD, see Materials and Methods) and monitoring the reduction rate at 465 nm. NADH made in exactly the same way (with H_2O in place of D_2O) and authentic NADH were used as controls. The C-D bond has a lower zero-point energy than

the C-H bond, due to the greater mass of the deuterium atom, and subsequently more energy is required for dissociation of the C-D bond. If bond breakage is rate-limiting to reduction of protein C, then the reduction might be expected to be slower with NADD in place of NADH. No difference was seen at lower concentrations of the NADD or NADH, but there did appear to be a significant difference in the rate of reduction at higher levels of the coenzyme. With NADD, the first-order rate constant was 1.5 times less than the first-order rate constant for NADH (both at 56.5 micromolar), at around 98 sec^{-1} c.f. 147 sec^{-1} respectively. Consistently smaller transmittance changes on reduction were seen with NADH, which would be expected from the more rapid rate constant, leading to greater reduction within the dead-time of the instrument. Therefore it seems that bond rupture could be partially rate-limiting for the transfer of the reducing equivalents from NADH to protein C, and the maximum rate for reduction of protein C by NADH (170 sec^{-1}) may be limited by this elemental step. However, since reduction of protein C with saturating NADH is at least 1.75 times faster than the most rapid of the NADH-acceptor reductase assays (with ferricyanide as the electron acceptor, see 4.2.), no appreciable NADD kinetic isotope effect would be expected for these assays (see 4.2.), and none was found, supporting the conclusion drawn in 4.1.A(2) that reduction of protein C is not limiting to the maximum rates of these assays.

4.1.C. The interaction of apo-iron-sulphur protein C with NADH.

Protein C was prepared without the iron-sulphur centre or MMO activity (see 3.2.), but with full NADH-acceptor reductase activity (with DCPIP, cytochrome c, and ferricyanide as acceptors). This apo-iron-sulphur protein C (final concentration 6.7 micromolar) was reacted with NADH (11.25 micromolar), monitoring reduction at 465 nm and 580 nm, and otherwise identical conditions to those used for native protein C. At 465 nm the rate of reduction was the same as that seen with native protein C (at the same concentration of NADH), and was complete within 100 msec, with the absorbance increasing on oxidation, as for native protein C. Observation at 580 nm showed a rapid but small absorbance increase appearing at the same rate as the 465 nm absorbance decrease, indicative of semiquinone formation. On oxidation, the absorbance increased to a maximum, and then decreased to the fully oxidized level, indicating that the apo-iron-sulphur protein C had been rapidly and strongly reduced by a stoichiometric excess of NADH (past the maximal semiquinone stage, see 3.1.B.2.), in the same way as occurs for native protein C.

The dependence of the reduction rate (monitored at 465 nm) on the NADH concentration was very similar to that of native protein C (see 4.1.A(2).), indicating that a similar kinetic mechanism holds for these two types of protein C, with respect to interaction with NADH. Therefore

this result is consistent with the maintenance of NADH-acceptor reductase activities by apo-iron-sulphur protein C (see 3.2.), and is good evidence that the iron-sulphur centre is not required for the oxidation of NADH by protein C, since its absence has no effect on the rate of reduction of protein C, and is consistent with the flow of reducing equivalents passing from NADH to the FAD prosthetic group, and thence to the iron-sulphur centre (see 4.3.B.), as indicated by the destruction and reconstitution studies in 3.2.

4.1.D. The interaction of protein C with NADPH.

The ability of a large excess of NADPH (56.5 micromolar) to reduce protein C (5.65 micromolar) was examined by stopped-flow at 465 nm. The first-order rate constant for reduction was $0.12 \pm .002 \text{ sec}^{-1}$, very roughly three orders of magnitude slower than the rate constant for reduction with the same concentration of NADH. Therefore NADPH reduces protein C much less readily than NADH. Colby and Dalton (1979) reported that the K_m for NADPH in the DCPIP acceptor-reductase assay was 15.5 mM, so the level of NADPH used in this experiment may be far below the saturation concentration for NADPH. The ratios of the k_{cat} / K_m for the two coenzymes (which for the purposes of comparison of the relative activity of protein C with the two separate coenzymes may be considered to be an apparent second-order rate constant at substrate concentrations away from saturation) are around 3000, indicating that NADPH is a

less active electron donor than NADH in this reductase assay. This stopped-flow experiment indicates that one of the reasons for the greater activity of protein C with NADH may be the greater rate of reduction of protein C by NADH as compared to that found with an equal concentration of NADPH.

An interesting observation by Colby and Dalton (1976) is that NADPH gives half the rate seen with NADH for the MMO activity. This small rate difference may be rationalised with the large difference in rates that would be seen for NAD(P)H-acceptor reductase assays at this concentration of coenzyme, because the MMO maximum reaction rate (3.7 sec^{-1} on a protein C basis) is very much slower than the maximum rate of reduction of protein C by NADH, 170 sec^{-1} , c.f. greater than or equal to 4.3 sec^{-1} for NADPH (estimated from the maximum activity seen with DCPIP as an acceptor, Colby and Dalton, 1979). Thus, although the maximum rate for protein C reduction with NADH is very much faster than that with NADPH, both these steps are faster than the rate-limiting step for the MMO reaction. This explains the relatively small difference in MMO rate seen for the two coenzymes, in spite of the difference in the rates of protein C reduction, because protein C reduction may be far from rate-limiting to the MMO reaction.

4.2. The independent acceptor reductase activities of protein C.

Steady-state kinetics may be used to investigate the acceptor reductase activities of protein C (Colby and Dalton, 1979), and to yield information on the interaction

of NADH with protein C. Unlike transient kinetics or the static spectroscopic examinations of protein C (see Chapter 3), these investigations require only catalytic amounts of protein C, making the technique extremely economical in comparison to techniques where the redox centres of protein C are examined directly.

4.2.A. The NADH-acceptor reductase activities of protein C

(1) Protein C (around 50 nmolar in 2 ml of 20 mM sodium phosphate buffer, pH7.0) reduction of the electron acceptors ferricyanide, DCPIP, cytochrome c, and oxygen, was examined by spectrophotometry under anoxic conditions, using NADH as the electron donor (see Colby and Dalton, 1979, and Materials and Methods). The maximum initial rates (with 200 micromolar NADH, at 45°C) can be quite variable, but were similar to those found previously, and are given in Table 4.1. It may be of interest that apo-iron-sulphur protein C appeared to have only half the NADH-oxidase activity of native protein C, indicating that the iron-sulphur centre may participate in the transfer of electrons to oxygen, although the product of this activity is unknown. The faster rates place a lower limit on the rate of reduction of protein C by NADH at 77 sec^{-1} , which is consistent with the rate constant for the reduction of protein C obtained from transient kinetics at 170 sec^{-1} (4.1.A(2).). The DCPIP and ferricyanide assays appeared to be first-order processes, as indicated by the linearity of plots of $\log [\text{DCPIP}]$ and $\log [\text{ferricyanide}] \longrightarrow \text{time}$, but the cytochrome c reaction was

Table 4.1. Rates for the NADH-acceptor reductase activities of protein C.

| acceptor | rate (micromole/min/mg C) | Molar extinction coefficient ($M^{-1}cm^{-1}$)* |
|--------------|------------------------------|---|
| DCPIP | 30-60 | 21,000 (600 nm) |
| cytochrome c | 130-180 | 19,100 (550 nm) |
| ferricyanide | 230-260 | 1,020 (410 nm) |
| oxygen | 0.014 | 6,220 (340 nm) |

*For cytochrome c this value is the increase in extinction coefficient on reduction, ferricyanide and DCPIP have negligible absorbance at the wavelengths concerned, on reduction, and NADH has negligible absorbance at 340 nm on oxidation, for the NADH-oxidase assay.

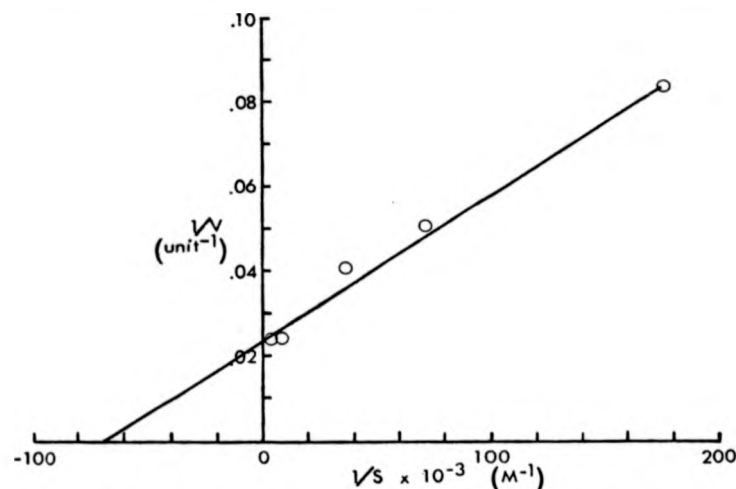
not first order. The DCPIP assay was used for further work because previous work (Colby and Dalton, 1979) was carried out with this dye as an electron acceptor. This assay is conveniently slow for accurate quantitation of the rate with direct addition of protein C (typically, protein C was added in a 1 microlitre Hamilton syringe, to initiate the assay:- the cuvette contents were stirred during the assay by means of a micro-flea). A minor disadvantage of this assay is the small amount of endogenous activity seen with DCPIP and NADH, which necessitates a small correction when calculating

initial rates. All three assays, DCPIP, ferricyanide, and cytochrome c, have always had parallel activities in native protein C and apo-iron-sulphur protein C, and the author has never resolved these activities, e.g. found a protein C with DCPIP activity but lacking cytochrome c activity. This result may be compared with that for adrenodoxin reductase where ferricyanide is reduced independently of adrenodoxin, but adrenodoxin is required for cytochrome c reduction (Lambeth and Kamin, 1977). Unlike apo-iron-sulphur protein C, the FAD group of adrenodoxin reductase requires the iron-sulphur centre of adrenodoxin to reduce cytochrome c.

4.2.B. Catalytic parameters of the NADH-DCPIP reductase activity of protein C

The K_m for NADH and k_{cat} for the DCPIP reaction were found by measuring the initial rates of DCPIP (22.5 micromolar) reduction with 5.65-283 micromolar NADH, initiating the reaction with 0.049 nmoles of protein C. The initial rate was corrected for endogenous activity at the higher level of NADH concentration, but endogenous activity was otherwise negligible in comparison to the rates seen in the presence of protein C. A Lineweaver-Burk plot gave a linear slope (see Figure 4.4.), indicating that this reaction obeys the behaviour predicted by the Michaelis-Menten equation, with a k_{cat} of 25 sec^{-1} (calculated from a specific activity of 41.3 units) and a K_m for NADH of 14 micromolar. The range in K_m values seen with this assay is 10-20 micromolar, in moderate agreement with

Figure 4.4. Catalytic parameters for NADH, with DCPIP as acceptor, of protein C.



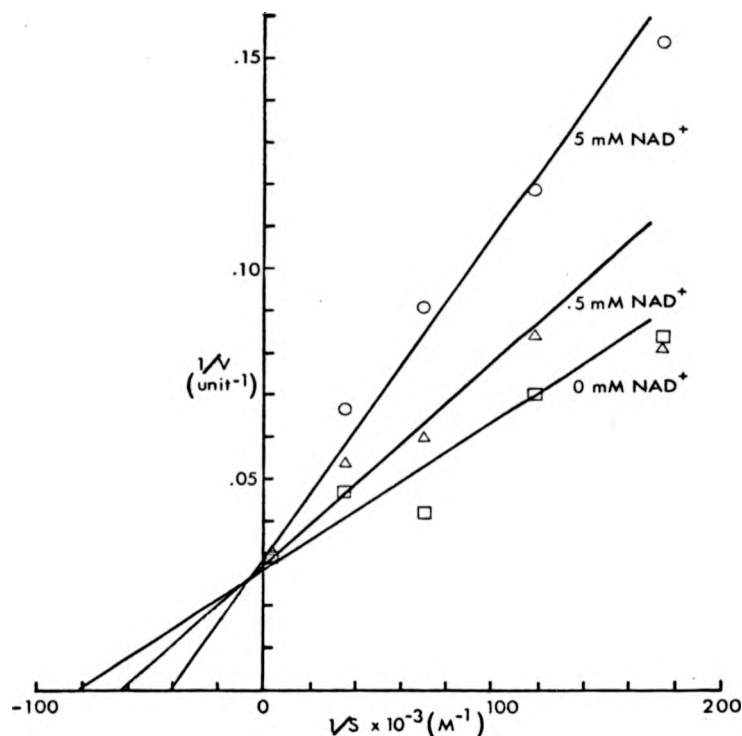
Protein C (0.049 nmoles) was used to catalyse reduction of DCPIP (22.5 micromolar) in the presence of 5.65–283 micromolar NADH, in 2 ml of 20 mM sodium phosphate pH7.0 at 45°C, under anaerobic conditions.

the value found previously, at 50 micromolar (Colby and Dalton, 1979). This value is in close agreement with the K_b for NADH at 20 micromolar, estimated from stopped-flow experiments (see 4.1.A(2).), and indicates that for protein C interaction with NADH, the Michaelis-Menten constant is a dissociation constant, and that protein C is in rapid equilibrium with NADH with respect to binding, followed by a relatively slow electron transfer step from NADH to protein C. A K_m of 10 micromolar was found for NADH with

apo-iron-sulphur protein C, again using DCPIP as the electron acceptor. Together with the similar k_{cat} to that of native protein C, these kinetic data indicate that apo-iron-sulphur protein C interacts with NADH in a very similar way to that seen with native protein C, and is consistent with a flow of electrons from NADH to the FAD redox centre (not requiring the iron-sulphur centre), and then to the iron-sulphur centre of protein C.

The interaction of NAD^+ with protein C was examined using the DCPIP assay as detailed in this section, but in the presence of 0 mM, 0.5 mM, and 5 mM NAD^+ . The Lineweaver-Burk plots of the results (see Figure 4.5.) show that NAD^+ seems to be a competitive inhibitor of the NADH-DCPIP activity, since the lines intersect near the y-axis, and a plot of the apparent inhibition constant against the concentration of NAD^+ gives an x-axis intercept of 5 mM, in agreement with qualitative inspection of Figure 4.5. NAD^+ is therefore a fairly poor competitive inhibitor of NADH interaction with protein C in this assay, indicating that NAD^+ binds protein C only fairly loosely in comparison to NADH. Protein C may be at least semi-reduced during the assay, so that this K_i is a measure of the affinity of semi/reduced protein C for NAD^+ . A stopped-flow experiment was carried out with a stoichiometric excess of DCPIP (11 micromolar protein C, 5.65 micromolar NADH and 21 micromolar DCPIP), monitoring the reaction at 465 nm and 600 nm. The transmittance traces showed that considerable protein C reduction had occurred with this excess of DCPIP,

Figure 4.5. Competitive inhibition of the NADH-DCPIP activity of protein C by NAD^+ .



Lineweaver-Burk plots for the NADH-DCPIP activity of protein C in the presence of 0, 0.5, and 5 mM NAD^+ intersect on the y-axis, suggesting that NAD^+ is a competitive inhibitor of NADH interaction with protein C.

indicating that protein C could be at least semi-reduced during steady-state turnover in this assay, and therefore this state of reduction might also hold in the much slower MMO reaction. This conclusion seems reasonable because the rate constant for reduction of protein C is faster than the fastest NADH-acceptor reductase assay.

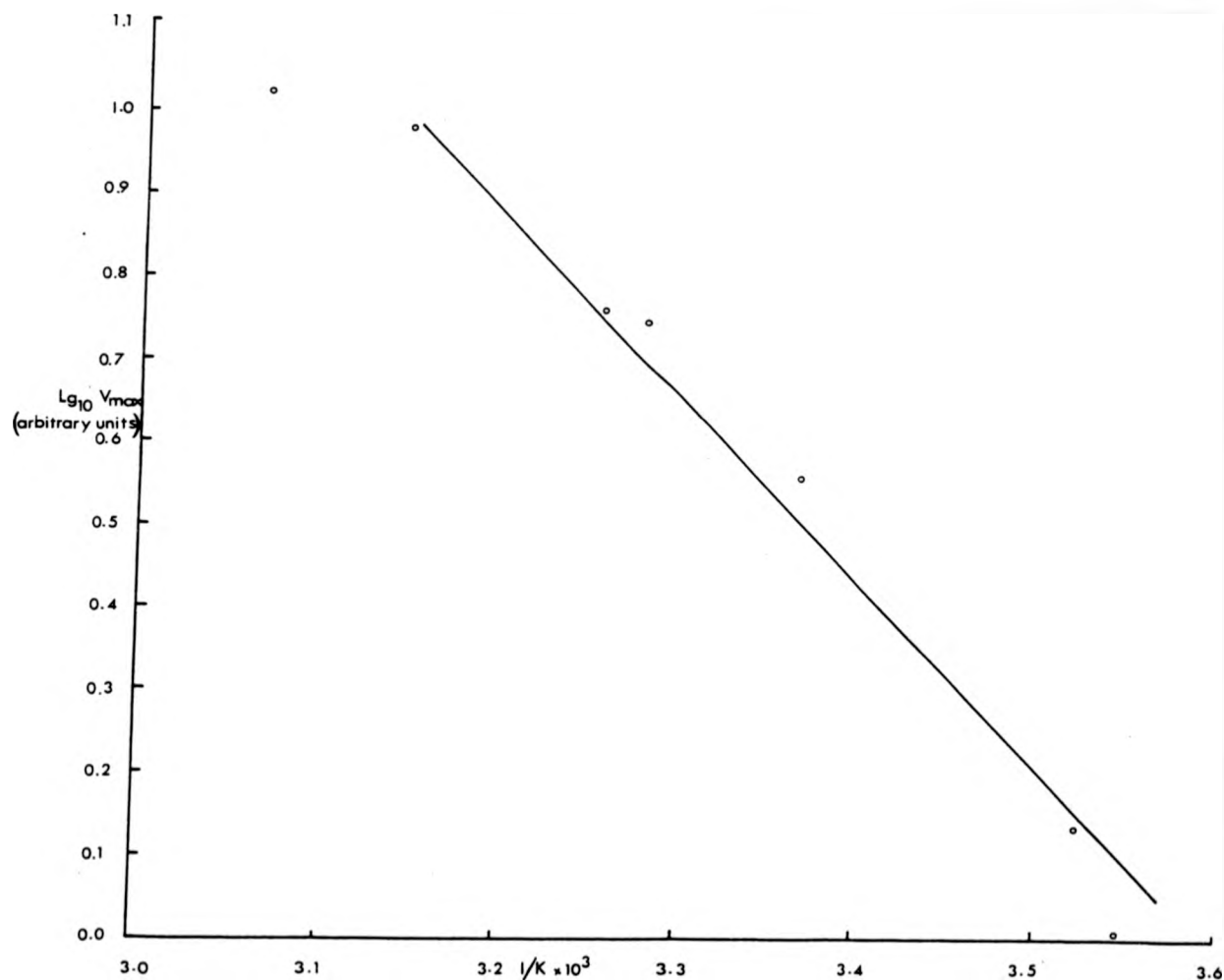
The putative loose binding of NAD^+ to semi/reduced protein C would be consistent with the ability of NADH to rapidly and strongly reduce protein C past the 2-electron stage as indicated by stopped-flow (4.1.A(2).), and with the lack of significant stabilization of any protein C mid-point potentials in the presence of NAD^+ (see 3.1.D.).

4.2.C. The variation of k_{cat} for the NADH-DCPIP activity of protein C

Attempts have been made to probe the rate-limiting step(s) on protein C for the reductase activity seen with DCPIP, by examining the initial maximum steady-state rate of the reaction under a variety of conditions. The NADH-DCPIP assay was carried out at a range of temperatures between 9°C and 53°C (in 20 mM phosphate buffer), monitoring the temperature by means of a temperature probe adjacent to the sample cuvette, in the cell holder. Reactions were started by the addition of protein C to DCPIP which had been placed in the cell holder for 10 minutes beforehand. The maximum rate of the reaction increased with temperature, and an Arrhenius plot ($\log k_{\text{cat}} \longrightarrow 1/T$) gave a linear slope from which the activation energy for the reaction was $10 \text{ kcal mole}^{-1}$, Fig. 4.5B.

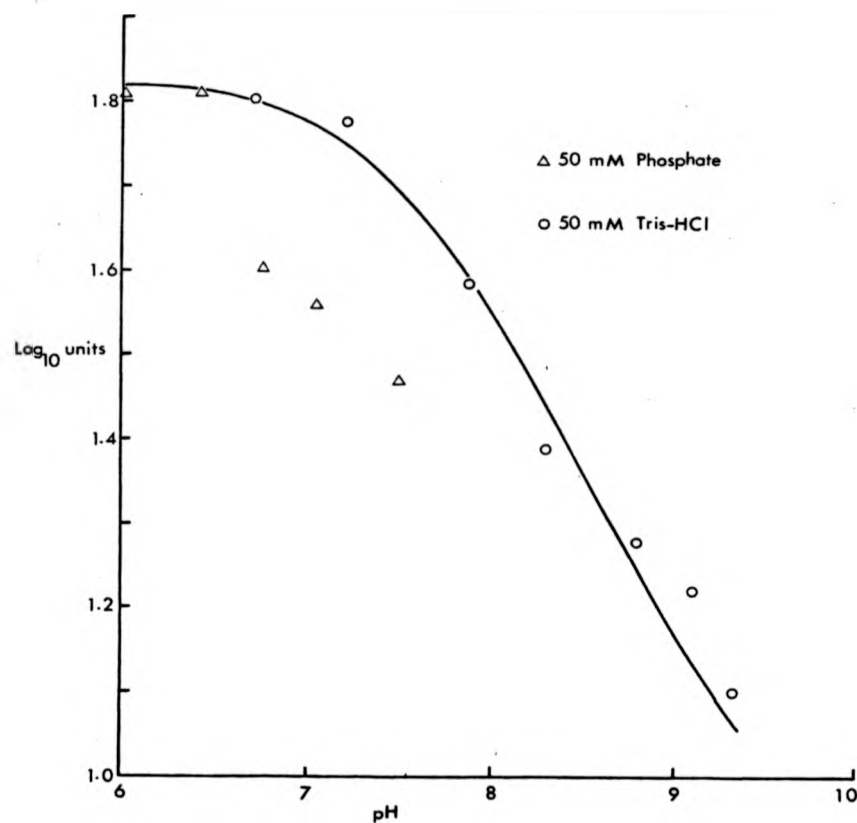
The pH profile of protein C maximal activity was found (at 45°C), using a range of sodium phosphate and Tris-HCl buffers (50 mM) to set the pH between 6 and 9.4 (measured at 45°C). The rate was corrected for a decrease in DCPIP absorbance seen at the more acidic pH values. The results indicated that acidic form(s) of the reactants are most active (see Figure 4.6.), with the highest activity being

Figure 4.5(B). The temperature dependence of V_{max} for the NADH-DCPIP activity of protein C.



Conditions were as for Figure 4.6. The specific activity for the protein C was 40 micromoles DCPIPH₂ minute⁻¹ mg⁻¹ protein. The Arrhenius activation energy is given by the slope / 2.3 × gas constant.

Figure 4.6. The pH dependence of the NADH-DCPIP activity of protein C.



Protein C (0.0715 nmoles) was added to initiate reduction of 0.17 micromoles of DCPIP by 0.23 micromoles of NADH in 2 ml of 50 mM Tris-HCl or 50 mM sodium phosphate buffer, at 45°C under anaerobic conditions.

seen at less than pH7. It may be that there is a catalytic group on protein C with an apparent pK_a of above 7. The pH dependence seen here is contrary to the report of Colby and Dalton (1979), who found the optimal pH to be 8.5-9.0 for this NADH-DCPIP reaction, although the optimal pH seen here is in a similar range to that found for the MMO reaction, at pH6.5-7.0 (Colby and Dalton, 1976).

Protein C activity for DCPIP reduction seems to be independent of the ionic strength, as indicated from the similarity of the rates seen in 0 M NaCl and 0.5 M NaCl, and this suggests that not all the reactants involved in the rate-limiting step of the reaction are charged (it may be of interest that the MMO reaction is inhibited by salt, M.P.Woodland, personal communication).

The NADH-acceptor reductase assays appear to be affected by the presence of D_2O , see Table 4.2.

Table 4.2. Effect of 90% D_2O on NADH-acceptor reductase activities of protein C.*

| acceptor | k_H/k_D |
|--------------|-----------|
| DCPIP | 1.0-1.3 |
| ferricyanide | 1.5-2.0 |
| cytochrome c | 1.8-2.0 |

*In 25 mM sodium phosphate buffer, pH 7.0.

The DCPIP assay displays the lowest solvent isotope effect, which may be related to its relatively slow rate as compared to the other assays. A solvent isotope effect may be indicative of a rate-limiting hydrogen atom transfer (e.g. Lambeth and Kamin, 1979).

However, the reduction of protein C by excess NADH (monitored by stopped-flow, see 4.1.) is unaffected by the presence of D_2O (90% v/v), indicating that the rate-limiting step for the reductase reaction (with saturating NADH) may lie after reduction of protein C by NADH on the reaction pathway, and is not the reduction step, a conclusion consistent with the different rate constants for these reactions. The reduction step appears to have another property which is different to that of the NADH-DCPIP activity, in that the reduction step is less sensitive to pH variation than the NADH-DCPIP activity (undergoing a 1.4-fold decrease in maximum rate from pH7 to pH9, c.f. a 3-fold decrease over the same pH range).

In summary, the rate-limiting step for the NADH-acceptor reductase activities of protein C does not appear to be the reduction of protein C by (saturating) NADH, and lies after this step on the reaction pathway, perhaps explaining why different rates are seen with different substrates. For the NADH-DCPIP activity, the rate-limiting step (with saturating NADH), appears to depend on a protonated group (acidic form) of apparent pK of circa 7.

4.2.D. The interaction of protein C with reduced benzyl viologen and NAD⁺

The ability to run the MMO off an alternate source of reducing power to NADH, such as a redox dye or a redox electrode, might be an interesting technological proposition. In addition, there is interest in systems capable of generating NADH from NAD⁺ (e.g. Payen *et al.*, 1983; for a review see May and Padgett, 1983), since only catalytic amounts of coenzyme might be required in the presence of an NADH-generating system, perhaps forming part of a biosensor for any NADH-utilizing enzyme. In this experiment, benzyl viologen was semi-reduced with dithionite (in 0.25 M Tris-HCl buffer, pH7.6), under anoxic conditions.

Separate addition of excess NAD⁺ (1.5 mM) had no effect on the absorbance of the benzyl viologen, but on the addition of a catalytic amount of protein C, the benzyl viologen absorbance at 550 nm underwent a large and rapid absorbance decrease at an initial rate of 17 micromoles dye minute⁻¹ mg protein C⁻¹ ($E_{550} = 8,100 \text{ M}^{-1} \text{ cm}^{-1}$). At 340 nm a peak was observed which was air stable, unlike benzyl viologen, and was removed rapidly on the addition of DCPIP under aerobic or anaerobic conditions. If the peak was NADH, then the concentration of NADH formed was approximately 40 micromolar. This had been formed from around 90 micromolar benzyl viologen (after correction for some air oxidation). Therefore it appears that the benzyl viologen ($E_m = -349 \text{ mV}$) is able to reduce protein C, which can in turn reduce NAD⁺. That NADH is formed is evidenced by the increase in absorbance at 340 nm, where NADH is known to absorb, and by

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the rapid disappearance of this peak on addition of DCPIP, due to the NADH-acceptor reductase activities of protein C. Thus it seems possible to run protein C "backwards". It would be interesting to examine the ability of protein C to interact with ferrocene, because this reagent interacts well with redox electrodes and is not readily oxidized by oxygen:- raising the possibility of running the MMO off an electrode. The rate of production of NADH from NAD^+ would be expected to be strongly inhibited by NADH accumulation, probably with a K_i of around 20 micromolar (the dissociation constant of protein C for NADH, see 4.1.A(2).), and this could limit its potential use.

4.3. The interaction of protein C with protein A

Preliminary work has been carried out on the interaction of pure protein C with pure protein A and pure protein B. All of this work has been carried out in collaboration with Dr. M.P.Woodland, who has purified and characterised these two latter proteins. There are two major problems which have limited these investigations, namely the general instability of the three proteins, and the very large amount of time and effort required to prepare the three proteins, which has resulted in experiments being limited by the availability of the MMO components. In addition, the type of experiments undertaken here require a detailed knowledge of the individual properties of the MMO components, if the results are to be interpreted usefully. We have attempted to probe electron transfer between protein

C and protein A by simple equilibrium and kinetic techniques (both transient and steady-state), and to investigate the role of protein B. Neither protein A nor protein B have significant absorbance at wavelengths greater than 300 nm, and neither of these two proteins undergoes any significant absorbance change on reduction (no prosthetic group has been detected in protein B; M.P.Woodland, personal communication). This lack of absorbance change means that protein C absorbance changes may be examined directly, in the presence of proteins A and B, and are not confused by absorbance changes from the other two MMO components. Since the absorbance contributions of the redox centres in protein C are reasonably well characterised, examination of the MMO components through these redox centres seemed a good place to begin; investigating the flow of reducing equivalents from NADH to protein A, and whether protein B is required for this process.

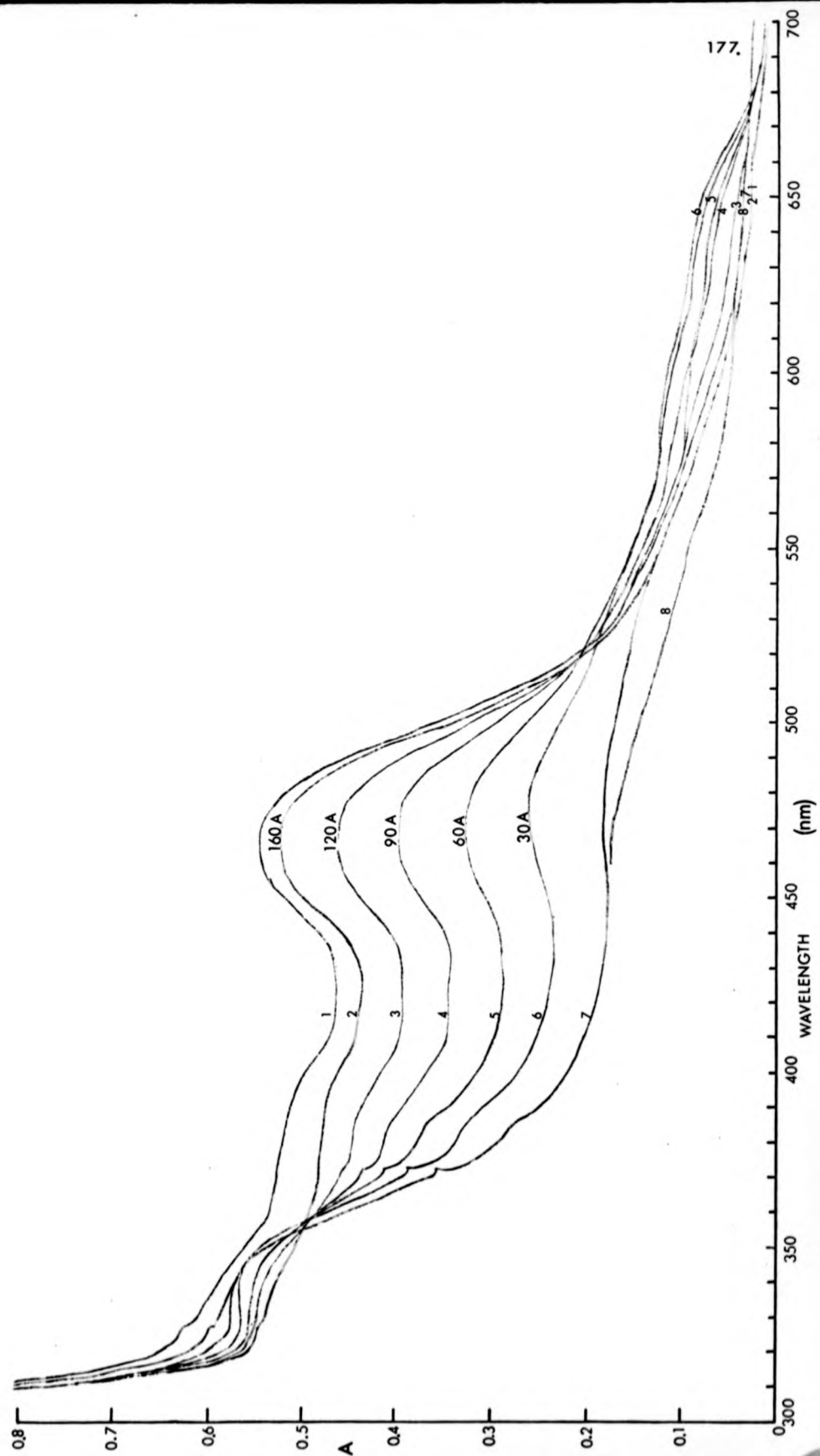
4.3.A. Equilibrium electron transfer between protein C and protein A

Protein C (90 nmoles, in 1.4 ml) was nearly fully reduced with NADH in the anaerobic cuvette (see Materials and Methods), and the extent of reduction was monitored spectrophotometrically. Addition of 0.16 ml of anaerobic protein A (25 nmoles), added in small aliquots with a gas-tight Hamilton syringe, was required to fully oxidize the protein C, see Figure 4.7. Addition of an aliquot of anaerobic buffer during the oxidation gave no oxidation of

Figure 4.7. Equilibrium electron transfer between protein C and protein A.

Protein C (90 nmoles) was reduced with near stoichiometric NADH in the anaerobic cuvette. The reduced protein C was then reoxidised by the addition of 25 nmoles of protein A in aliquots (in a total volume of 160 microlitres), giving a final volume of 1.56 ml. The partial scan, extending from 460 nm to 700 nm, is the protein C fully reduced by buffered dithionite.

protein C, indicating that the oxidation effect is due to protein A. The absorbance changes on oxidation of protein C were rapid (but not always immediate, occasionally taking 10-20 seconds to complete) and stable in between additions. Oxidation was judged to be complete after the addition of the protein A because no further change in the absorbance spectrum occurred on addition of ferricyanide or exposure to air. The stoichiometry on a polypeptide basis (mean of three experiments) was around 2.6 protein C molecules per protein A molecule, although the range in stoichiometry was quite large, between 2 and 3.7. Protein C peak relations appeared to be similar to those seen in the absence of protein A, and the semiquinone form is present, although appreciable decay of the iron-sulphur centre seems to have occurred, as indicated by the increased 340 nm : 465 nm ratio over non-reduced protein C (this loss of intensity at 395 nm and 465 nm, for example, is not reproducible, and may be minimal, and might be due to loss of sulphide from the iron-sulphur centre).



However, this experiment does demonstrate that protein A can accept electrons from reduced protein C under equilibrium conditions, and is capable of total oxidation of protein C. This result confirms the reports, in review format, that reduced protein C may be oxidized by protein A (Colby et al., 1979; Dalton, 1980). Further evidence that electron transfer takes place on an equilibrium basis comes from an E.P.R. experiment, where protein A exhibits its full signal within 10 seconds of NADH addition, in the presence of catalytic amounts of protein B and protein C (protein A cannot be reduced directly by NADH, as indicated by the lack of appearance of the $g = 1.934$ signal). The signal decreased after this time, perhaps due to further reduction of the putative anti-ferromagnetically spin-coupled iron centre in protein A (M.P. Woodland, personal communication). Electron transfer on an equilibrium basis is consistent with estimates of the mid-point potentials of the two proteins, since none of the mid-point potentials of the protein C redox groups are greater than the mid-point potentials of protein A. The stoichiometry for electron transfer from this experiment is in approximate agreement with estimates of the stoichiometry on a redox centre basis, assuming that protein C accepts around 1.2 ± 0.4 electrons per polypeptide (3.1.B.2.) and protein A has around 2.3 ± 0.7 irons on a protein basis (Woodland and Dalton, 1983).

4.3.B. A stopped-flow investigation of electron transfer
between protein C and protein A

(A) Aerobic stopped-flow was carried out, reacting protein C (final concentration 11.25 micromolar) with NADH (final concentration 5.65 micromolar), in the presence of proteins A and B (both 12.5 micromolar), with the conditions as detailed in 4.1.A. the presence of proteins A and B did not appear to affect the rate of reduction of protein C (at 465 nm), which was similar to that seen with protein C and NADH only, see Figure 4.8. Similarly, the maximal specific activity of protein C for the steady-state NADH-DCPIP assay is unaffected by the addition of stoichiometric amounts (relative to the amount of protein C) of proteins A and B to protein C. Therefore it appears that the MMO reaction may be "random order" for NADH binding and reduction of protein C. However, oxidation of protein C was very much more rapid in the presence of protein A and protein B, than in its absence, with a first-order rate constant of $1.7\text{--}4.6\text{ sec}^{-1}$, much faster than the protein A MMO activity at 0.19 sec^{-1} (estimated from the protein A MMO specific activity at 0.05 units), indicating that electron transfer between proteins A and C may not be the rate-limiting step of the MMO reaction (in the presence of excess reduced protein C). The oxidation of protein C appears to be uniform, and is accomplished much more rapidly than the rate-limiting step for the MMO reaction, on a protein A specific activity basis.

Figure 4.8. Electron transfer between protein C and protein A by aerobic stopped-flow.

Proteins A, B, and C (all 12.5 micromolar final concentration) were reacted in various combinations with NADH (5.65 micromolar final concentration) in 20 mM sodium phosphate pH7.0. Protein C was monitored at 465 nm, with a photomultiplier supply voltage of -420 to -430 V. V_{ox} corresponds to the fully oxidised protein C absorbance and V_{red} to the fully reduced protein C absorbance. The experiments were carried out under aerobic conditions at room temperature.

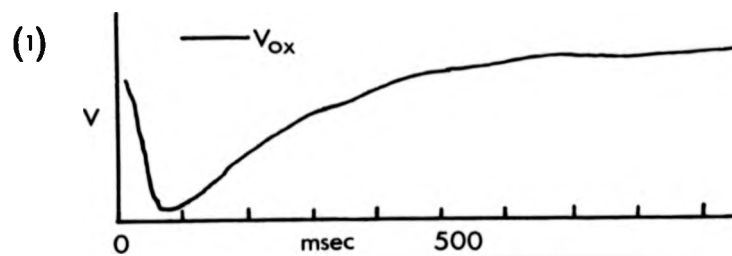
(1) Proteins A and B and NADH were reacted with protein C, at 0.5 V full scale and 1 sec full scale.

(2) Protein A and NADH were reacted with protein C, at 0.5 V full scale and 0.5 sec full scale.

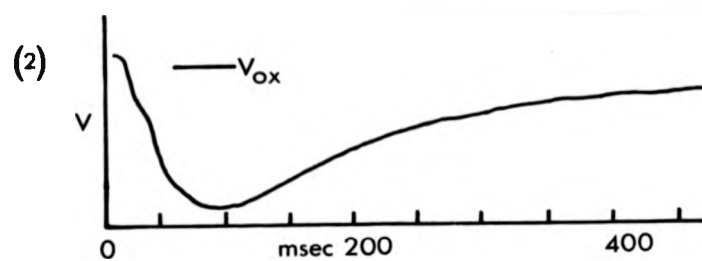
(3) Proteins A and B and NADH, were reacted with apo-iron-sulphur protein C (9.3 micromolar final concentration), at 0.2 V full scale and 0.5 sec full scale.

(4) Protein B and NADH were reacted with protein C, at 0.5 V full scale and 0.5 sec full scale.

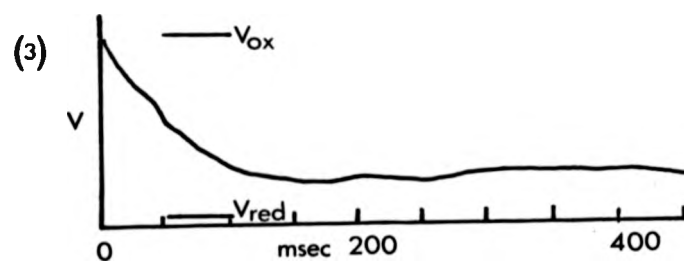
A+B+C



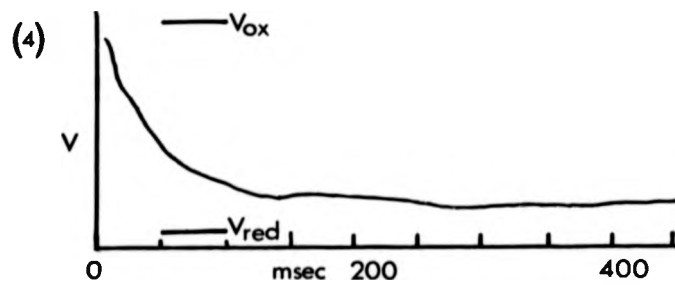
A+C



A+B + apo-FeS-C



B+C



At 465 nm both the quinone and the iron-sulphur centre make major reducible absorbance contributions, and the magnitude of the oxidation suggests that both redox centres must be involved. Therefore both redox centres may be reduced faster than the rate-limiting step for protein C MMO activity (and overall MMO activity), and both redox centres are oxidized at a rate equal to the rate-limiting step for protein C MMO activity, satisfying the criteria for their reduced states being intermediates.

The rate constant for electron transfer is in the range of the maximal activity for protein C MMO activity, at 3.7 sec^{-1} (based on a specific activity for protein C in the MMO assay of 6 units), which suggests that electron transfer to protein A is the rate limiting step for protein C in the MMO reaction. A similar rate constant for electron transfer is seen in the absence of protein B, indicating that protein B is not necessary for electron transfer between protein C and protein A. An initial result had suggested that protein B might be necessary for electron transfer between protein C and protein A, but this result was not confirmed in successive experiments. Therefore protein B may well act after the electron transfer step to effect the MMO reaction, because protein B is a definite requirement for MMO activity:- the protein A and protein C used here gave very low to negligible MMO rates in the absence of protein B, as was reported for the purified components by Dalton, (1980). Protein B would not accept electrons from protein C, since no oxidation occurred after reduction of protein C by NADH in

the presence of 12.5 micromolar protein B, as shown in Figure 4.8(4). The same electron transfer rate was seen whether the proteins A, B, and C were preincubated and reacted with NADH, or whether protein C was reacted with proteins A and B together with the NADH, suggesting that association of protein C with protein A is unlikely to be rate-limiting to the electron transfer reaction, with the concentration of MMO components used here (protein C and protein A appear to bind with high affinity, see 4.3.C.).

The iron-sulphur centre of protein C seems to be required for this electron transfer to protein A, because no oxidation of apo-iron-sulphur protein C (9.3 micromolar) was seen (Figure 4.8(3).), in the presence of proteins A and B at the stated concentrations, although reduction was seen at the same rate as occurs with native protein C. But the excess of NADH to protein C may make this experiment equivocal, although the excess of protein A might be expected to ensure some reoxidation of protein C, and no reoxidation was seen.

(B) Preliminary observations of the semiquinone form of protein C at 580 nm and 630 nm indicated the rapid formation of small amounts of semiquinone, formed at the same rate at which the 465 nm transmittance decreased, but which did not then decay. This might be due to stabilization of the semiquinone form by the other MMO proteins, or the presence of a dead end intermediate, e.g. some apo-iron-sulphur protein C in the native protein C preparations (although the presence of rapid semiquinone formation in apo-iron-sulphur

protein C indicates that these protein C molecules can also interact, since odd-electron forms are present after interaction with the 2-electron donor, NADH). Several of this type of short electron transport pathway have been proposed to operate on a 3-electron / 1-electron cycle. In these systems, the acceptor FAD takes 2 electrons from NADPH while in a fully oxidised form, and then transfers 1 electron to the iron-sulphur centre in oxidised form (or FMN in a semiquinone form), with the iron-sulphur centre undergoing 1-electron transitions and providing for electron transport to the final acceptor at a uniform potential (for a review of the P-450 reductases and other reductases thought to participate in a 3e/1e cycle, see Kamin and Lambeth, 1982). Thus, the kinetic stabilization of the semiquinone form of protein C would not be unlikely, and it could be an active intermediate in the catalytic cycle of the MMO, as suggested by Colby and Dalton (1978). An experiment required to prove this would be to react 1, 2, and 3-electron forms of reduced protein C with protein A, and monitor the kinetic competence of the various forms.

(C) However it must be noted that there is another explanation of the ability of protein A to cause rapid oxidation of protein C, namely that protein A merely enhances the NADH-oxidase activity of protein C by acting specifically on the iron-sulphur centre of protein C. But this explanation seems unlikely because protein C can cause a rapid appearance of the protein A E.P.R. signal,

indicative of bona fide electron transfer, and also because the rate constant for protein C oxidation is in the same range as the maximum rate for the protein C MMO reaction. To solve this problem, oxidation of reduced protein C by protein A would have to be demonstrated by stopped-flow under anaerobic conditions, and this experiment has only been done under equilibrium conditions. Rapid-freeze E.P.R. would be an admirable technique for this purpose because the protein A and protein C E.P.R. signals of interest have very different power saturation properties, and so may be resolved easily by this technique (M.P.Woodland, personal communication).

Another limitation here is that although electron transfer appears to have been demonstrated between protein C and protein A, these experiments have not been performed in the presence of an MMO substrate, such as cyanomethane. With the excess of protein A over protein C in these experiments, less than a single turnover of protein A might be expected, but it is not possible to rule out a small amount of protein A turning over rapidly and repeatedly with the reduced protein C, because protein A and protein C in combination have a steady-state NADH-oxidase activity (identified by Dr.M.P.Woodland, by NADH disappearance at 340 nm), which is up to 37-fold greater than the endogenous NADH-oxidase activity of protein C. This NADH-oxidase activity is unaffected by the presence of protein B (as is electron transfer by stopped-flow), and has a rate-constant of about 0.19 sec^{-1} (calculated from the maximal specific

activity on a protein A basis, for NADH disappearance). This rate constant is slower than the rate constant for electron transfer from protein C to protein A at 3.7 sec^{-1} , indicating that electron transfer from protein C to protein A may not be rate-limiting to the uncoupling activity. However on a protein C basis the specific activity for uncoupling is around 3 units to 5.3 units, a similar value to that found for the MMO-C specific activity, and indicates that electron transfer to protein A is also rate-limiting for protein C for the uncoupling activity. No uncoupling (i.e. the transfer of reducing equivalents from NADH through to protein A and oxygen independently of the organic substrate) is seen with apo-iron-sulphur protein C and protein A, indicating that the iron-sulphur centre is necessary for this activity. Uncoupling activity is also seen in the P-450 system, where it is present at all times, even in the presence of substrate (Coon and White, 1980). For the MMO system, addition of 2 mM cyanomethane and protein B causes up to a 2-fold enhancement of the NADH-oxidase activity (M.P.Woodland, personal communication), but the MMO activity was stimulated 11.6-fold (propylene oxide assays). Therefore protein B appears to couple electron flow (from NADH to oxygen) to the (net) insertion of oxygen into substrate, and it seems likely that protein B acts on protein A.

In summary, it appears that protein C can transfer electrons to protein A more rapidly than the rate-limiting step for the MMO reaction, and that neither protein B nor

hydrocarbon substrate is required for this step, which is probably the rate-limiting step for protein C MMO activity. The iron-sulphur centre is required for this step, indicating that the iron-sulphur centre is responsible for electron transfer from protein C to protein A. Protein B would then have to act "after" this step in the MMO reaction. Protein B is small, and unstable in the presence of proteases, and the MMO is the first enzyme on the methane utilization pathway, so could be an ideal candidate for regulation. Protein B might divert (couple) protein A away from uncoupling activities towards the production of methanol, and would then act at the site proposed by Dalton (1980), coupling electron flow to the (net) insertion of oxygen into methane.

4.3.C. Binding studies on protein A and protein C

The binding of protein C to protein A was examined using XM100A ultrafiltration membranes (Steinhardt and Reynolds, 1969). These membranes have a molecular weight cut-off of 100,000, so should exclude protein A (with a molecular weight of 220,000) on a molecular sieve basis, but allow protein C (37,000 molecular weight) to pass through the membrane. If protein C binds protein A, it will not pass through the membrane. Protein C was assayed above and below the membrane by MMO assays. The data were corrected for apparent lack of partitioning of protein C through the membrane, about 75-85% of the expected amount of protein C passes through the membrane in the absence of protein A.

Prior to this experiment, it had been shown that 22.7 nmoles of protein A could halt quantitatively the passage of 28 nmoles of protein C through the membrane, and that 9.1 nmoles of protein A could retard most of the protein C activity. The control for these experiments was to see if protein C would partition through the membrane before and after individual experiments, and in between experiments. This control was necessary because occasionally, no protein C would partition through the membrane, even in the absence of protein A, and this effect was irreversible. Therefore, experiments were performed with successively smaller amounts of protein A, so that any lack of partitioning of protein C would show up. For the experiment here, protein C (28 nmoles) was incubated with 0 to 22.5 nmoles of protein A in a total starting volume of 1.7 ml, at 4°C (20 mM Tris-HCl pH7, 5 mM sodium thioglycollate), dismantling and washing the ultrafiltration cell and membrane between each of the separate incubations. After incubation for 10 minutes with stirring, 1.2 ml of the sample was passed (the dead-volume of the cell outlet was 0.3 ml), following which 200 microlitre samples of the filtrate and the supernatant were assayed for MMO-C activity. The experiment was in agreement with previous results, so that at the highest protein A concentration protein C appeared to be totally bound, see Table 4.3. The results may not be treated by a Scatchard plot, because the concentrations of both proteins were changing throughout these experiments, adding different amounts of protein A and with different amounts of protein C being retained. However

Table 4.3. Protein C binding to different concentrations of protein A.

| [protein A] micromolar | [C _{total}] | [C _{free}] | C _{bound} A ⁻¹ * |
|---------------------------|-----------------------|----------------------|--------------------------------------|
| 0 | 16.5 | 16.5 | 0 |
| 5.6 | 19.2 | 15 | 0.746 |
| 11.25 | 28.2 | 10.8 | 1.55 |
| 22.5 | 22.2 | 1.5 | 0.92 |
| 56.2 | 23.1 | 0 | 0.41 |

*Moles of protein C bound per mole of protein A.

it is possible to estimate the amount of protein C bound for each concentration of protein A, by subtraction of the amount of protein C in the filtrate from the amount of protein C in the supernatant, see Table 4.3. Inspection of this Table indicates that the maximum stoichiometry for the binding of protein C to protein A is around 1.6 protein C molecules per protein A molecule, although these data are not satisfactory inasmuch as there is a considerable non-systematic variation in the total amount of protein C present in the supernatant, C_{total}. One definition of the K_D is the concentration of free ligand at half-saturation of the binding agent. Protein A appears to be very roughly half-saturated at 22.5 micromolar protein A, when the concentration of free protein C was 1.5 micromolar,

indicating that protein C binds to protein A with high affinity. It may not be a coincidence that the non-linear dependence of MMO activity seen at low concentrations of soluble extract occurs at very roughly 1 micromolar protein C (Colby and Dalton, 1976, who suggested that this property might be expected from a multi-component system; J.Lund, personal observation). This finding may not be inconsistent with the linear response seen at low concentrations of protein C in MMO-C assays (Colby and Dalton, 1978; J.Lund, personal observation), because in these assays proteins B and A are present in large excess, and not in the stoichiometric amounts used in these experiments. Under these conditions association of the MMO components may not be rate-limiting to the MMO reaction, but protein A MMO assays still show this non-linear dependence even in the presence of excess protein B and protein C.

The estimate of the stoichiometry of binding of protein C to protein A is in reasonable agreement with the stoichiometry found by anaerobic spectrophotometric titration (around 2.6, see 4.3.A.), and also with the stoichiometry estimated by NADH-oxidase assays, titrating protein C with protein A and vice versa, which indicated that the optimal uncoupling specific activity was obtained with 2 and 1-2 protein C molecules per protein A molecule respectively, on a protein basis (M.P.Woodland, personal communication). It may be that protein C dissociates from protein A at some stage of the catalytic cycle, perhaps after electron transfer, because the specific

activities of these two proteins for MMO activity are different, namely around 6 units for protein C and about 0.05 units for protein A, and after correction for differences in molecular weight there is still a 20-fold difference in specific activity between protein C and protein A. One simple explanation of this discrepancy might be that one protein C can shuttle around several protein A molecules, and this would be consistent with both the reduction of protein C and electron transfer to protein A being appreciably quicker than the rate-limiting step.

CHAPTER 5. General Summary

The methane monooxygenase present in soluble extracts of Methylococcus capsulatus (Bath) can be resolved into three components, called A, B, and C.

Component C is thought to be the reductase component of the methane monooxygenase (MMO), and NADH the reducing substrate. Component C may transfer reducing equivalents to component A, thought to be the component responsible for oxygenation of the hydrocarbon substrate. Component C (or protein C) is a polypeptide chain with one FAD and one Fe_2S_2 centre per polypeptide, purified to homogeneity by a DEAE-cellulose column chromatography step, a Sepharose-4B-AMP column step and a Sephadex G-100 column step (Colby and Dalton, 1978 and 1979).

The aim of this project was to investigate the transfer of reducing equivalents between the substrate NADH and each of the two redox centres in protein C, and electron transfer between protein C and protein A. The preparation and basic characterisation of protein C (a), a necessity for mechanistic studies, is discussed in Chapter 2. The equilibrium properties of the redox centres of protein C are investigated in Chapter 3 (b), along with evidence for the order of electron flow through the two redox centres, from NADH to protein A. Chapter 4 is concerned with the interaction of protein C with NADH and protein A, mainly on a kinetic basis (c).

(a) Modifications were introduced to the published procedure for growth of M. capsulatus (Bath) in the 100 litre

fermenter, namely the use of a small and active inoculum, carefully controlled pO_2 values, the inclusion of 5 mM sodium thioglycollate in all buffers, and rapid processing to produce soluble extract (2.1.A.). The purification scheme for protein C was also made more rapid, due to the instability of protein C. The resultant procedure gives useful amounts of pure protein C in a reproducible manner. Routinely, protein C is purified 100-fold with 30-40% yield, in a total time of 10.5-11 hours (2.2.A.). Growth on ethene-contaminated methane appears to delete all three methane monooxygenase components in a fairly specific way, further indicating that protein C is a true component of this soluble enzyme system (2.1.B.). Present work on the basic properties of protein C is in good qualitative agreement with previous work, and indicates that protein C is an acidic (2.3.A.3.) monomer (2.3.A.2.) of molecular weight 37,000 (2.3.A.1), but differs in some important respects, namely the stability of this enzyme (2.3.C.), and stoichiometries of the redox centres on a protein basis (2.3.B(2).). However, it does seem very likely that the occupancy of protein C by its redox centres is in the 0.4:1 to 0.8:1 range, and that the redox centres are present in equal amounts. Further studies are required into the cause of this putative loss of redox centres, the cause of protein C instability (protein C MMO activity decays at between 1% and 5% $hour^{-1}$), and how these problems might be overcome.

(b) The optical contributions of the two redox centres of protein C appear to have been resolved, using mersalyl to destroy the iron-sulphur centre (3.1.A.). The iron-sulphur centre seems to be very much like that of spinach ferredoxin, by its E.P.R. and optical properties, and the semiquinone appears to be a neutral semiquinone. Absorbance spectra of protein C on reduction (3.1.B.) and E.P.R. spectra on reduction (3.1.C.) allow the ordering of the three mid-point potentials, and indicate that protein C can exist in 1 (semiquinone), 2 (reduced iron-sulphur and semiquinone), and 3 (reduced iron-sulphur and dihydroquinone) electron forms, consistent with the role of protein C as an electron pair splitting enzyme. The mid-point potentials of protein C, at -150, -220, and -260 mV, are shown to be consistent with its ability to pass electrons onto protein A, which has mid-point potentials at around +150 and -150 mV (3.1.D.), although these studies were done on protein C in the absence of the other components. Selective removal and reconstitution of the two redox centres indicates that the iron-sulphur centre is not necessary for initial reduction of protein C by NADH (3.2.), since apo-iron-sulphur protein C has similar catalytic parameters to native protein C for reduction of the protein and interaction with alternate electron acceptors (4.1.C., 4.2.B.). The iron-sulphur centre is necessary for electron transfer to protein A (4.3.B.), since methane monooxygenase activity is greatly reduced in its absence, and correlates with the presence of this redox centre (3.2.1.). It would

be interesting to perform reconstitutions of this centre with exogenous constituents, as can be done for the FAD group (3.2.2.). This evidence is consistent with electron flow proceeding from NADH through the FAD to the iron-sulphur centre and then to protein A.

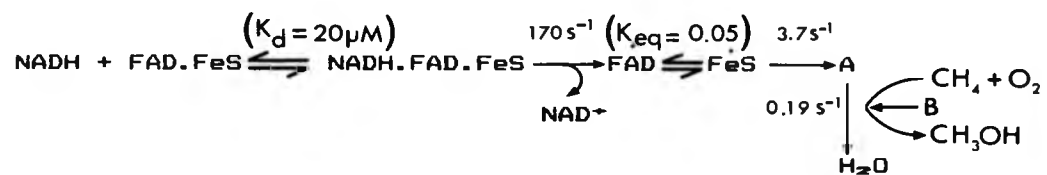
(c) Both redox groups of protein C appear to be prosthetic groups, since they may be reduced by NADH, and reoxidised by protein A, faster than the rate-limiting step of the overall methane monooxygenase reaction (By stopped-flow, 4.1.A(1)., 4.3.B.), and electron transfer to protein A would appear to be the rate-limiting step with respect to protein C activity in this enzyme system. Protein B is not required for electron transfer to protein A (4.3.B.), and would appear to act after this step to couple the flow of reducing equivalents to the production of methanol from methane (M.P. Woodland, personal communication, 4.3.B.).

A gross kinetic mechanism for protein C interaction with NADH and protein A may be proposed. For protein C interaction with NADH, the reaction is consistent with a rapid equilibrium for the binding of NADH to protein C, with a K_D of 10-20 micromolar (4.1.A(2).), such that the K_M is a dissociation constant (4.2.B.). Reduction of both redox centres appears to occur with a rate constant of 170 sec^{-1} (4.1.A(2).), faster than any of the enzymic activities of protein C (4.1.D.), and the two redox centres might be expected to be in equilibrium since electron transfer to protein A is slow compared to their rates of interconversion

(4.1.A(2).). Rupture of the C-H bond may only be partially rate-limiting to reduction of protein C by NADH, since no large kinetic isotope effect was seen with NADD in place of NADH (4.1.B.). NAD^+ may be presumed to exit rapidly from reduced protein C since the protein may be reduced to the 3-electron form quickly (4.1.A.), and NAD^+ binds to protein C only loosely (4.2.B.). Electron transfer to protein A proceeds at $1.7\text{--}4.6 \text{ sec}^{-1}$, and is in the same range as the specific activity of protein C for methane monooxygenase activity, indicating that electron transfer to protein A is the rate-limiting step for protein C (4.3.B.). This step is much faster than the overall enzymic rate (0.19 sec^{-1}), so electron transfer is probably not rate-limiting to the overall MMO reaction. The evidence here is consistent with a role for protein C as a $2e^-/1e^-$ transformase, splitting electron pairs from NADH. NADH would be expected to interact with the FAD group, which then transfers electrons to the iron-sulphur centre for passage to protein A in 1-electron steps at a constant potential (3.2.). The catalytic competence of the various reduced forms of protein C remains to found.

(d) Possible uses of protein C might be as an NADH regeneration system (4.2.D.), or as part of a sensor for NADH and NADPH (3.1.E.), a major advantage of this protein being that there would be no extra substrate requirements, or unwanted products, because protein C alone should interact with the cofactors, and a suitable source or sink of electrons.

Figure 5.1. Proposed kinetic scheme for electron transfer in the soluble MMO.



CHAPTER 6. Materials and Methods

Growth and harvesting of *Methylococcus capsulatus* (Bath)

Methylococcus capsulatus (Bath) was grown for 20 hours at 45°C in batch culture on a nitrate/mineral salts medium (Whittenbury *et al.*, 1970, see Table 6.1) in a 100 litre fermenter (L.H. Engineering Ltd., Stoke Poges, Bucks, U.K; Colby and Dalton, 1976; Colby *et al.*, 1977), with stirring (to increase the gas transfer rate) at approximately 500 R.P.M. throughout the 100 litre run.

Table 6.1. Mineral salts medium used for growth of *M. capsulatus* (Bath).

| mineral salts | concentration |
|----------------------------------|------------------|
| KNO ₃ | 19.80 mM |
| MgSO ₄ | 4.07 mM |
| CaCl ₂ | 1.80 mM |
| trace elements | |
| Fe-EDTA | 10.35 micromolar |
| FeSO ₄ | 1.80 micromolar |
| CuSO ₄ | 0.80 micromolar |
| ZnSO ₄ | 1.39 micromolar |
| H ₃ BO ₃ | 0.24 micromolar |
| CoCl ₂ | 0.21 micromolar |
| Na ₂ MoO ₄ | 2.07 micromolar |
| EDTA | 0.67 micromolar |
| MnCl ₂ | 0.10 micromolar |
| NiCl ₂ | 0.04 micromolar |
| buffer (pH 6.8) | |
| Na ₂ HPO ₄ | 2.33 mM |
| KH ₂ PO ₄ | 1.91 mM |

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Dalton, 1977). For serial 100 litre runs, 80 litres of culture was spun down, and washed by resuspension in 5 litres of ice-cold 20 mM sodium phosphate buffer, containing 5 mM sodium thioglycollate (a stabilizing agent for protein C) and again spun in the same ice-cooled Westphalia continuous centrifuge. The remaining 20 litres of culture was made to 100 litres with nitrate/mineral salts medium and was stabilised within 2 hours, the cells then batching up in preparation for the next harvest. After the washing step the cells were resuspended in about 1 litre of ice-cold 20 mM Tris-HCl buffer, containing 5 mM $MgCl_2$ and 5 mM sodium thioglycollate, and broken by decompression in a French press. Crude bacterial extracts were prepared by DNase 1 treatment to reduce the viscosity of the extract, followed by a single passage of the whole-cell suspension through an ice-cooled French pressure cell (Aminco, Silversprings, Maryland, U.S.A.) at $> 13,000$ P.S.I., then made up to 1.2 or 1.5 litres with the same buffer. To produce the soluble extract, the crude extract was centrifuged at 80,000 g for 1 hour in a 6 x 300 ml rotor, giving a clear red supernatant which was decanted from the pellet and immediately frozen by drop-wise addition into liquid nitrogen, followed by storage at $-70^\circ C$ (Colby *et al.*, 1977). A typical time from harvesting of the 100 litre fermenter to storage of the soluble extract would be about 6 hours. Growth and harvesting of Methylococcus capsulatus (Bath) was always carried out in collaboration

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with G.Chapman, due to the very large amount of labour (required in a short space of time) involved in these processes.

Purification of protein C of the methane monooxygenase

The methodology used to purify protein C to an acceptable level of homogeneity was as follows:- All procedures were carried out in 20 mM Tris-HCl pH7 (4°C), 5 mM sodium thioglycollate at 4°C. Approximately 200 ml of crude extract (prepared with 5 mM sodium thioglycollate) was loaded onto an 11 x 4.5 cm column of DEAE-cellulose, equilibrated with buffer, at circa 400 ml hour⁻¹. The column was then washed with a 200 ml portion of buffer (collecting fraction A), then with a 200 ml of buffer containing 0.1 M NaCl, followed by 300-400 ml of buffer with 0.2 M NaCl (fraction B), until the unidentified purple band seen with thioglycollate is near the bottom of the column, when fraction C is eluted in 40-50 ml with buffer containing 0.5 M NaCl. The fraction C was immediately ultrafiltered to around 15 ml on a PM30 Amicon membrane (exclusion limit 30,000 molecular weight), and drop-frozen in liquid nitrogen. The purification time from crude extract was 4.5-5 hours. Purification was monitored by SDS-PAGE analysis and MMO-C assays, detecting epoxyethane by gas chromatography using a flame ionization detector and a porapak Q (Waters Associates) column.

Two 15 ml fraction C lots were combined, thawed and pumped at 60 ml hour⁻¹ onto a 1.5 x 11 cm column of fresh 5'AMP-Sepharose 4B (Pharmacia Fine Chemicals)

equilibrated with buffer. The column was washed with about one half a column volume of buffer containing 0.5 M NaCl to reduce protein-protein electrostatic interactions, followed by one column volume of buffer, and the protein C which was still on the column was chased off the column with 1 mM ethanol-free NADH in buffer. Protein C was then drop-frozen into liquid nitrogen, with a total purification time of around 6-6.5 hours, from soluble extract.

Preparation of an Anti-protein C IgG affinity chromatography column

An anti-protein C antibody affinity column was constructed as follows:- 40 ml of rabbit anti-protein C antiserum (prepared by G.Chapman) was diluted to 160 ml with distilled water to lower its ionic strength, and then loaded onto a 170 ml DEAE-cellulose column equilibrated with 0.01 M phosphate buffer pH8. After incubation for 1 hour to allow binding of proteins, 160 ml of IgG was washed off with 0.01 M phosphate buffer pH8, and ultrafiltrated on an XM100A Amicon membrane (exclusion limit 100,000 molecular weight) until the protein concentration was 4 mg ml⁻¹ by the Bio-Rad protein assay (BIO-RAD Laboratories). This anti-protein C IgG preparation was pure as indicated by SDS-PAGE (in 8 M urea, after reduction with 2-mercaptoethanol and alkylation with iodoacetate) on the LKB 2117 Multiphor system (7.5% acrylamide gel). The anti-protein C antibody was then coupled to cyanogen bromide activated Sepharose-4B. 2 g of dry CNBr-activated-Sepharose 4B was treated with 200 ml of 1mM HCl (in a column),

then with 15 ml of coupling buffer, 0.1 M NaHCO_3 , 0.5 M NaCl pH8.3, and added to 2 ml IgG (11 mg/ml) in 14 ml of coupling buffer and shaken gently for 2 hours at room temperature. The gel was transferred to coupling buffer plus 0.2 M glycine at pH8.0 overnight at 4°C. Excess adsorbed protein was removed by washing with the coupling buffer followed by 0.1 M acetate buffer (0.5 M NaCl pH4.0) and then again with coupling buffer (Affinity chromatography booklet, Pharmacia Fine Chemicals). 2 ml of swollen immunoaffinity gel was placed into a pasteur pipette column with a glass wool plug. 1.4 ml of fraction C was loaded and washed on with 0.2 ml of buffer, then incubated for 30 minutes to allow protein binding. The column was washed with 5 column volumes of buffer, and yellow protein C then eluted with 8 M urea.

Assay of protein C MMO activity

The specific activity of the protein C for MMO activity in various states of purification was measured, using the BIO-RAD protein assay and ethylene oxide production from ethene by gas chromatography with an F.I.D. (Colby et al., 1977). Ethylene oxide is volatile at room temperature and pressure so precautions were taken in preparing ethylene oxide standards. A Finn-pipette was cooled to -20°C, then 10 microlitres of neat ethylene oxide added to 100 ml of cold water in a 100 ml bottle. This 2 mM ethylene oxide was pipetted immediately into freezing vials and stored in a liquid nitrogen cryostat. Standards were thawed immediately before use and injected directly into a gas chromatograph equipped with a flame ionization detector.

MMO assays

In this assay, methane monooxygenase activity was estimated by the production of epoxyethane from ethene (Colby *et al.*, 1977). The assays were carried out in conical flasks (7 ml internal volume) containing 1 ml of reaction mixture and sealed with Suba Seal stoppers. Reaction mixtures (1 ml) contained: 20 mM sodium phosphate buffer, pH 7.0; 5 mM NADH; and 3 ml of ethene (gas phase), together with around 5 mg of soluble extract. For assays of the protein C activity (MMO-C assays), excess fraction A (3.4 mg) and fraction B (1.3 mg) were used (Colby and Dalton, 1978), together with around 0.04 mg of pure protein C or 0.12 mg of fraction C. For whole cell assays, 1 ml of cells was incubated with 10 mM formate, to supply reducing power instead of NADH, but otherwise identical conditions. The flasks were incubated at 45°C in a shaking water bath (90 oscillations minute^{-1}) for 3 minutes, when NADH was added to initiate the assay. 5 microlitre samples of the solution were removed at appropriate time intervals (*e.g.* 0, 3, and 6 minutes) and injected into the gas chromatograph. The chromatograph was calibrated with freshly thawed 2 mM epoxyethane standards (stored in a liquid nitrogen cryostat), and epoxyethane production was calculated from the peak height (Colby *et al.*, 1975). Activities were calculated from the amount of epoxyethane produced with time.

The gas chromatograph was a Pye series 104 gas chromatograph (Pye Unicam, Cambridge, U.K.) fitted with a

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The gas chromatograph was a Pye series 104 gas chromatograph (Pye Unicam, Cambridge, U.K.) fitted with a

flame ionization detector. Samples were injected into 2.1 metre x 3.2 mm internal diameter glass columns packed with Porapak Q (Waters Associates, Milford, Massachusetts, U.S.A.), operated at 180°C, with nitrogen carrier-gas flow rates of around 50 ml minute⁻¹.

Protein assays

Protein C was assayed by the Bio-Rad protein assay, the method of Lowry and a direct (precipitation) method.

In the precipitation method, protein C with a known absorbance at 465 nm was precipitated with 20% trichloroacetic acid, then microcentrifuged. After several washings with acetone/water (50:50 v/v), the protein was vacuum dried and weighed on a calibrated 5-figure balance. All supernatants were pooled, neutralised and concentrated, and gave zero protein content using the Bio-Rad protein assay.

In the Bio-Rad protein assay (Bio-Rad Protein assay booklet, BIO-RAD laboratories, 1979), 0.1 ml of the protein to be assayed (at 0-1.4 mg ml⁻¹) is added to 4.9 ml of 5 x diluted and filtered Bio-Rad reagent (a Coomassie dye which undergoes a wavelength shift to 595 nm on binding to the hydrophobic areas of proteins). After 10 minutes the samples were read at 595 nm against a range of bovine serum albumin (BSA) standards (0.1 ml of 0-1.4 mg ml⁻¹ BSA with 4.9 ml of 5 x diluted Bio-Rad reagent). The BSA had been weighed previously on a calibrated 5-figure balance.

For the method of Lowry, samples of protein C (0-1 mg) were read against 0-1 mg ml⁻¹ BSA standards. Samples and standards were treated with 0.1 ml of 5 M NaOH solution and made up to 1 ml with water, then boiled for 5 minutes and cooled. 2.5 ml of a solution of 5% sodium carbonate, (with 0.02% copper sulphate, and 0.04% sodium potassium tartrate, added before use) was added to the samples and standards, which were then left for 10 minutes. 0.5 ml of diluted Folin-Ciocalteu reagent (1 N acidity) was added, and the solution vortexed immediately. After 30 minutes, the absorbance was read at 750 nm (Kennedy and Fewson, 1968).

NADH-acceptor reductase assays

These assays were performed in a similar way to that described in Colby and Dalton (1979). 2 ml of 20 mM sodium phosphate buffer pH7, containing 0.9 micromole ferricyanide, or 0.19 micromole of DCPIP, or 0.05 micromole of horse heart cytochrome c, was placed in a 3 ml cuvette and made anoxic by sparging with helium for 10 minutes. Anoxia was maintained by means of a Suba Seal, which allows injection of the assay components NADH (0.23 micromole) and protein C (0.1 nmole). The cuvette was equilibrated for 10 minutes at 45°C in the SPB-200 spectrophotometer (Pye-Unicam), and the temperature was monitored using a temperature probe adjacent to the sample cell, and controlled using a Churchill chiller thermo circulator. The spectrophotometer has a stirring attachment for a micro-flea, so that the cuvette contents could be stirred

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rapidly, resulting in efficient mixing. After the addition of 0.23 micromole of NADH, the reaction was initiated by the addition of 0.1 nmole of protein C. The reactions were followed at 600 nm (DCPIP), 410 nm (ferricyanide) and 550 nm (cytochrome c), NADH-oxidase activities were monitored at 340 nm. Initial rates were calculated from the gradients during the fast phase of the reactions.

Preparation of ethanol-free NADH

2 g of NADH (B.D.H., grade 1) was dissolved in 20 ml of 20 mM sodium phosphate buffer, pH7 and placed in a separating funnel with 150 ml of diethyl ether (A.R. grade). After 5 cycles of shaking, consisting of removal of ether, and addition of fresh ether, the NADH solution was made up to 20 ml and evacuated, checking for ether and ethanol using the flame ionization detector with a porapak Q column. After removal of the ether, the solution was adjusted to around 100 mM NADH, knowing the E_{340} to be 6.2×10^3 . The NADH was then stored at -20°C in freezing vials.

Preparation of NADD

To 1 M NADH in 20 mM Tris-HCl pH7.6, 10 mg of sonicated beef-heart mitochondria was added. The solution was 4 ml of 90% D_2O (v/v), and any residual NADH-oxidase activities of the mitochondria were inhibited with 10 micromolar rotenone (solubilised in methanol). The sample was left in an anaerobic jar overnight at 4°C under "white spot" nitrogen, after which the mitochondrial fragments were

spun down. The NADD was precipitated with 4 volumes of cold ethanol, washed with cold ethanol, and freeze-dried for storage under dessication at 4°C. A control sample of NADH was prepared with 100% H₂O, but otherwise identical conditions. The method and the mitochondria were a gift from Dr. D.E.Griffiths.

Anaerobic titrations

The central apparatus is a special quartz cuvette designed by Prof. H.Dalton, see Figure 6.1., in which samples may be evacuated to remove solubilised oxygen, and then blanketed with anaerobic nitrogen, giving anaerobic samples. The redox agent is added by means of a 17.8 cm long-needled Hamilton gas-tight syringe. The needle pierces two gas-locked suba seals which are separated by an anaerobic gas-tight space. Dithionite ($E_{314} = 8000 \text{ M}^{-1} \text{ cm}^{-1}$) was used to estimate the rate of oxygen entry into this system at several nanomoles minute⁻¹. The redox agent is made anaerobic in an analogous gas-tight flask.

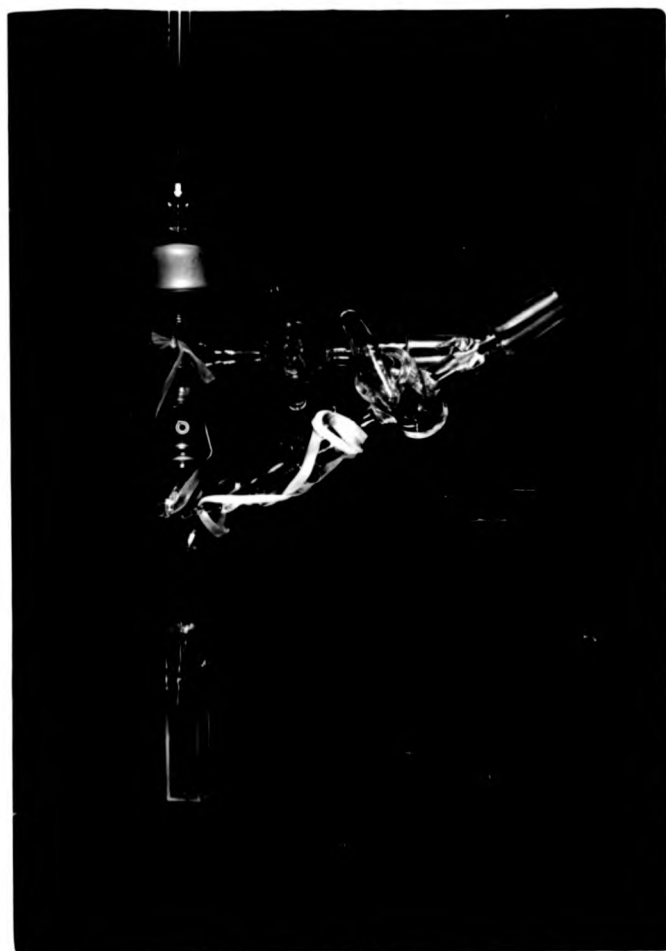
A dark box was constructed to accommodate the anaerobic cuvette in the Pye-Unicam SP8-200 spectrophotometer.

The dark box is removed to allow access to the anaerobic cuvette. Identical absorbances were obtained with either the dark box or the normal spectrophotometer lid over the instrument's wavelength range, indicating that the dark box is light-tight. A manual override switch was installed to allow activation of the photomultiplier even when the normal spectrophotometer lid was removed.

Figure 6.1. The anaerobic quartz cuvette.



Figure 6.1. The anaerobic quartz cuvette.



A vacuum line was modified to allow a high degree of anaerobicity to be attained. All rubber fittings were replaced by glass and glass-metal junctions. All taps and joints were liberally greased with Apiezon M high vacuum grease. A single stage rotary vacuum pump was used to develop a pressure of around 0.03 mm of mercury, as indicated by a McLeod gauge.

Anaerobic nitrogen was obtained by passing white spot nitrogen through a Nil-ox oxygen scrubbing system (Jencons (Scientific) Ltd., Beds.), and then to the vacuum line via oxygen-impermeable nylon tubing (Jencons). Nitrogen flow was set at 100 ml minute⁻¹ using a flow regulator prior to the Nil-ox apparatus. Converted gas sampling flasks were coupled to the vacuum line and used as dithionite and ferricyanide dispensers. The dispensers were gas-tight, with all glass fittings. Redox agents were removed via Suba Seals using gas tight Hamilton syringes.

This apparatus may be used rapidly and easily, and solutions were made anaerobic by 5 cycles of evacuation and flushing with nitrogen, taking less than 15 minutes. No frothing of protein C solutions was seen. Titrations were usually performed at 4°C (due to the instability of protein C) in 20 mM Tris-HCl pH7.0, using a small magnetic flea to ensure good mixing. The temperature was maintained at 4°C using a Churchill chiller-cooler with 20% ethylene glycol in aqueous solution. Titrations were performed as rapidly as possible, but ensuring that the system had stabilized in between

additions of redox agent, so that a set of titrations could be performed within 30-40 minutes. For the stoichiometric estimates, titrations were carried out with and without glucose oxidase (1 unit ml^{-1}) and 1 mM glucose, but no appreciable difference was seen, with NADH perhaps because of the relatively poor NADH-oxidase activities of protein C, so that the small amount of oxygen entering the system did not react with protein C.

Redox electrode studies

The redox electrode (Kent Industrial Measurements Limited) was a micro combination electrode consisting of a platinum sample electrode and a silver / silver chloride reference half-cell. To measure the redox potential, the electrode was linked to a millivoltmeter (Pye-Unicam model 290 pH meter) calibrated in 1 mV graduations. The electrode was calibrated with a ferrous ammonium sulphate / ferric ammonium sulphate standard and with the redox dye safranin O, during an anaerobic titration. Routinely before use, the electrode was cleaned and the resistance across the ceramic junction ascertained to be less than 25 Kilo ohms with an Avometer. Meter readings were converted to potentials relative to the standard hydrogen electrode by the addition of 200 mV to the meter reading. Spectrophotometric / redox electrode titrations were carried out with the redox electrode attached to the anaerobic cuvette system. For simple potentiometric titrations the redox electrode attachment was sealed off with a double Suba Sealed injection and sampling port.

Dansylation procedure

Dansylated marker amino acids were prepared by suspension of the amino acid (10 mM) in 0.5 M NaHCO_3 . 0.1 ml of this solution was reacted with 0.1 ml of dansyl chloride in acetone, to give an excess of 2 micromoles over all reactive groups. After 30 minutes the samples were diluted to 1 ml with 1% formic acid and stored in a stoppered bottle. Single labelled lysine and tyrosine derivatives were made by reacting a 2-fold excess of the amino acid with dansyl chloride. The final concentration of these markers was 0.5 mM (Stark, 1967).

0.5 mg of protein C in 50 microlitres was added to 0.1 ml of a saturated solution of NaHCO_3 , 0.35 ml of 8 M urea (deionized to remove cyanate and ammonia by passage through a column of Amberlite MB-1 mixed bed resin), and 0.5 ml of 15 mg/ml dansyl chloride in acetone (A.R.). The preparation was incubated at 37°C for 2 hours, and the protein precipitated by the addition of 1.3 ml of 10% trichloroacetic acid. After centrifugation the sample was washed with 0.5 ml of 10% trichloroacetic acid, followed by washing with acetone, and then vacuum-dried. The dry protein was hydrolysed by incubation with 0.1 ml of 6 M HCl at 105°C for 4 hours and 18 hours, after which the HCl was removed in a dessicator with NaOH pellets, and the hydrolysate redissolved in 10 microlitres of 1 M NH_3 for thin-layer chromatography (R. Offord, Oxford practical course sheets for School of Biochemistry, 1979).

Thin layer chromatography was performed on the hydrolysate and markers, on 0.25 mm x 20 x 20 cm plates of silica gel 60 (Merck), without a fluorescent indicator.

Samples were applied by spotting 10 microlitres of the protein hydrolysate and 2 microlitres of marker onto the plate. Chromatography was performed in 1-dimension in two solvents, recording the intermediate and final results under u.v. light. The two respective solvent systems were; benzene : pyridine : glacial acetic acid, 80:20:2 (v/v), and n-butanol saturated with 0.2 M NaOH (Niederwieser, 1972).

Ultracentrifugation of protein C

The sedimentation properties of protein C were examined by analytical ultracentrifugation using a Beckman model E ultracentrifuge. An An-D rotor with quartz cell windows and u.v. optics was used, running at 48,000 R.P.M. Protein C was present at a concentration of 32 micromolar, stabilised by 1.7 mM sodium thioglycollate and operating at 4°C, in the presence of 0.15 M NaCl to reduce ionic effects.

An 8.5 hour run was carried out with exposures taken manually, at about 30 minute intervals. The negatives were scanned with a Joyce-Loebel scanner at a 1:3 expansion.

Electrophoresis of protein C

Unless otherwise stated, electrophoresis was carried out on the LKB Multiphor (2117) flatbed system (Fehrström and Moberg, 1977). Denaturing electrophoresis was carried out with sodium dodecyl sulphate (1% w/v) in 0.1 M sodium phosphate buffer pH7.1, in the acrylamide gel and the electrode buffer. 10%, 7.5%, and 5% acrylamide gels (with 0.27%, 0.2%, and 0.135% w/v bisacrylamide

respectively), were made up using 0.07% ammonium persulphate (w/v) and 0.15% TEMED (v/v) to cause polymerisation, and left overnight to complete polymerisation. Samples (50 micrograms) were prepared by dilution with sample buffer (10 mM sodium phosphate buffer, pH 7.1, 1% SDS w/v, 1% 2-mercaptoethanol v/v) to 0.2 ml to dilute the salt, and acetone precipitated with 1 ml acetone. The sample was centrifuged, dried over nitrogen, and made up in sample buffer, followed by incubation at 80°C for 20 minutes. The sample was chilled and 2-mercaptoethanol (4% v/v) and bromophenol blue (enough to colour the sample) were added, to a total volume of 10 microlitres, followed by centrifuging to remove SDS and any precipitated protein. The gel had been pre-electrophoresed for 30 minutes at 150 mA. Samples were applied with a Finn-pipette and run at 20 mA for the first 10 minutes and then at 200 mA for around 4 hours, at constant current, and cooling the apparatus with water at ambient temperature. After electrophoresis, the gel was fixed in trichloroacetic acid (11.4% w/v) with sulphosalicylic acid (3.4% w/v) in a methanol water solution (0.43:1). The stain was Coomassie Brilliant Blue R-250 (0.25% w/v), in a solution of methanol (45% v/v), glacial acetic acid (9.2% v/v), and water to 100%. Gels were stained overnight and destained in ethanol (30% v/v), glacial acetic acid (10% v/v), and water to 100%. For estimates of molecular weight, negatives were scanned using a Joyce-Loebel scanner at a 1:9 expansion.

Non-denaturing electrophoresis (5% acrylamide,

0.135% bisacrylamide) was performed on protein C under stabilizing conditions, using the LKB flatbed system. The running conditions were 100 mM Tris-Glycine, pH 8.9, 5 mM sodium thioglycollate and 4°C, and the gel was run at 45 mA for 2 hours, followed by fixing and staining as above (Fehrstrøm and Moberg, 1977).

For the molecular weight estimation by the method of Lambert and Fine (1979), a vertical gel electrophoresis system was used (B.R.L.), with a 5% to 30% linear gradient of polyacrylamide (0.19% to 1.14% bisacrylamide), in Tris-Borate-EDTA buffer (1.075%, 0.504%, and 0.093% w/v respectively), pH 8.5. The gel was run at 60 V (constant voltage) at room-temperature. Samples (0.05 ml of desalted protein C in 10% v/v sucrose) were loaded at times 0, 1, 2, 3, 4, 5 and 6 hours, and then left for 2 hours, each sample being loaded simultaneously with adjacent low molecular weight markers. The gel was fixed and stained as detailed above.

Isoelectric focusing of protein C

Denaturing IEF was performed over a pH 4-7 gradient, using 10 cm cylinder gels and 8 M urea as the denaturant. Samples were prepared by dialysis against 10 mM Tris-HCl pH 7.4, 0.5 mM EDTA, 25 mM NaCl, and 0.5 mM 2-mercaptoethanol. Samples were made up to 8 M urea and 1% Nonidet P-40, 2.5% 2-mercaptoethanol and 1% Ampholines. The gels were 6% acrylamide 0.3% bis-acrylamide, 3.2% Ampholines and 8 M urea (Aristar). Gels were run for 5,600 volt hours

(13 hours), with 10 mM phosphoric acid at the anode and 20 mM NaOH (degassed) at the cathode, and stained with Coomassie Brilliant Blue after fixing and destaining to remove Ampholytes (S.Riordan, personal communication). Several gels were saved before fixing for sectioning and determination of the pH of the 5 mm gel section (macerated under anoxic conditions) in 2 ml of distilled water, by means of a pH electrode.

Non-denaturing IEF of protein C was carried out for 4 hours at 4°C over a pH range of 3.5-8.5. The gels were 3.2% acrylamide, 0.1% bisacrylamide (both pre-treated with Amberlite MB-1), with 2.3% Pharmalytes. The cathode solution was 0.01 M Histidine and the anode solution was 0.01 M Glutamate. The gels were run for 2000 volt hours and were stained for N.B.T. activity or Coomassie stained after fixing and destaining to remove Pharmalytes (Pharmalyte Instruction leaflet, Pharmacia Fine Chemicals, 1981).

Nitro Blue Tetrazolium stain

NADH-N.B.T. activities of protein C were shown by immersion of gels for 10 minutes in 0.5 mM NADH, 50 mM sodium phosphate pH7, with 10 mg of N.B.T. in a total volume of 10 ml. A positive stain showed up as a purple colouration (Colby and Dalton, 1979).

Chemicals

Most compounds, enzymes, substrates, products, media components, etc. were obtained from the following

manufacturers: B.D.H. Chemicals, Poole, Dorset, U.K.; Pharmacia Fine Chemicals, Mddx., U.K.; Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.; Aldrich Chemical Co. Ltd., Gillingham, Dorset U.K.; Kodak Ltd., Kirby, Liverpool, U.K.

Gases

Methane (technical grade), and ethene (CP grade) were obtained from the British Oxygen Co., London, U.K.

Photography

Photographs were taken with a Pentax SP 500 camera and Kodak Panatomic X or Agfaortho 25 film.

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